



COPY OF PAPERS  
ORIGINALLY FILED

1645  
RECEIVED

AUG 28 2002 #7/101

TECH CENTER 1600/2900 09-2502

Practitioner's Docket No. 70281/55,986

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: S. Yamamoto, et al.

Group No.: 1645

Application No.: 09/879,257

Filed: June 12, 2001

Examiner: Not Yet Assigned

For: HYBRID ENZYME AND USE THEREOF

Assistant Commissioner for Patents  
Washington, D.C. 20231

TRANSMITTAL OF CERTIFIED COPIES

Attached please find the certified copy of the foreign application from which priority is claimed for this case and verified translations thereof:

Country: Japan

Application Number: 2000 - 274219

Filing Date: September 8, 2000

Country: Japan

Application Number: 2000-174604

Filing Date: June 12, 2000

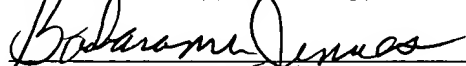
CERTIFICATE OF MAILING (37 C.F.R. SECTION 1.8a)

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Date: August 21, 2002

Barbaranne Jenness

(type or print name of person mailing paper)

  
Signature of person mailing paper

**WARNING:** *"When a document that is required by statute to be certified must be filed, a copy, including a photocopy or facsimile transmission of the certification is not acceptable." 37 C.F.R. section 1.4(f) (emphasis added).*

  
SIGNATURE OF PRACTITIONER

Reg. No.: 38,227

Cara Z. Lowen

*(type or print name of practitioner)*

Tel. No.: (617) 439-4444

Dike, Bronstein, Roberts & Cushman

Intellectual Property Practice Group

EDWARDS & ANGELL, LLP.

P.O. Address

Customer No.: 21874

P.O. Box 9169

Boston, MA 02209

**NOTE:** *"The claim to priority need be in no special form and may be made by the attorney or agent, if the foreign application is referred to in the oath or declaration, as required by section 1.63." 37 C.F.R. section 1.55(a).*



DECLARATION

RECEIVED  
AUG 28 2002  
TECH CENTER 1600/2900

I, Keita Takizawa, Wako Pure Chemical Industries, Ltd., Tokyo Office, 1-7, Nihonbashihoncho 2-chome, Cho-Ku, Tokyo, Japan, do hereby solemnly declare:

1. That I am acquainted with the Japanese and English language;  
and
2. That the English text attached hereto is a true translation of the following document:

Japanese Patent Application No. 174604/2000

AND I MAKE SOLEMN DECLARATION conscientiously believing the same to be true and correct.

This 24th day of July, 2002

K. Takizawa

Keita Takizawa



RECEIVED

AUG 28 2002

TECH CENTER 1600/2900

日 本 国 特 許 庁  
JAPAN PATENT OFFICE

別紙添付の書類に記載されている事項は下記の出願書類に記載されている事項と同一であることを証明する。

This is to certify that the annexed is a true copy of the following application as filed with this Office

出 願 年 月 日

Date of Application:

2000年 6月12日

出 願 番 号

Application Number:

特願2000-174604

出 願 人

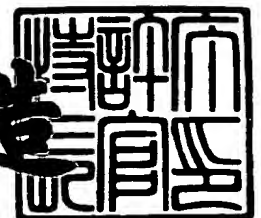
Applicant(s):

和光純薬工業株式会社

2001年 5月11日

特許庁長官  
Commissioner,  
Japan Patent Office

及 川 耕 造



出証番号 出証特2001-3038775



【書類名】 特許願

【整理番号】 WJ017

【あて先】 特許庁長官殿

【国際特許分類】 C12N 9/00  
C12N 15/52  
G01N 33/50

【発明者】

    【住所又は居所】 兵庫県尼崎市高田町 6 番 1 号 和光純薬工業株式会社  
                                大阪研究所内

    【氏名】 山本 幸子

【発明者】

    【住所又は居所】 兵庫県尼崎市高田町 6 番 1 号 和光純薬工業株式会社  
                                大阪研究所内

    【氏名】 城 実

【発明者】

    【住所又は居所】 兵庫県尼崎市高田町 6 番 1 号 和光純薬工業株式会社  
                                大阪研究所内

    【氏名】 花田 寿郎

【発明者】

    【住所又は居所】 兵庫県尼崎市高田町 6 番 1 号 和光純薬工業株式会社  
                                大阪研究所内

    【氏名】 小畠 伸三

【特許出願人】

    【識別番号】 000252300

    【氏名又は名称】 和光純薬工業株式会社

【代理人】

    【識別番号】 100089314

    【弁理士】

    【氏名又は名称】 大多和 明敏

【代理人】

【識別番号】 100086999

【弁理士】

【氏名又は名称】 大多和 曉子

【手数料の表示】

【予納台帳番号】 014742

【納付金額】 21,000円

【提出物件の目録】

【物件名】 明細書 1

【物件名】 図面 1

【物件名】 要約書 1

【プルーフの要否】 要

【書類名】 明細書

【発明の名称】 ハイブリッド酵素およびその用途

【特許請求の範囲】

【請求項 1】 配列番号 1 に示したアミノ酸配列の一部を含むペプチドにより一部が置換されるか、又は当該ペプチドが挿入されたアミノ酸配列を有するハイブリッド酵素であって、当該ハイブリッド酵素が、前記ペプチドが置換又は挿入される前と同様の酵素活性を有し且つ置換又は挿入されたペプチド部分に当該ペプチドに対して結合能を有する物質が結合すると当該酵素活性が変化する性質を有するハイブリッド酵素。

【請求項 2】 ペプチドが、配列番号 1 に示したアミノ酸配列のうちから選ばれた少なくとも 6 個以上の連続したアミノ酸配列を含むものである請求項 1 記載のハイブリッド酵素。

【請求項 3】 ペプチドが、C-反応性蛋白質と結合能を有する物質と結合可能な性質を有するものである請求項 2 記載のハイブリッド酵素。

【請求項 4】 ペプチドのアミノ酸配列が配列番号 2 ～ 5 の何れかの配列から選ばれた少なくとも 6 個以上のアミノ酸配列を含むものである、請求項 1 記載のハイブリッド酵素。

【請求項 5】 酵素が、グルコース-6-リン酸脱水素酵素、 $\beta$ -ガラクトシダーゼ又はアルカリホスファターゼである、請求項 1 記載のハイブリッド酵素。

【請求項 6】 ペプチドと結合能を有する物質が抗体である請求項 1 記載のハイブリッド酵素。

【請求項 7】 請求項 1 ～ 6 の何れかに記載された酵素を含んでなる、C-反応性蛋白質測定用試薬。

【請求項 8】 更に抗C-反応性蛋白質抗体を含んでなる、請求項 7 記載の試薬。

【請求項 9】 請求項 1 ～ 6 の何れかに記載された酵素を含む試薬を含んでなる、C-反応性蛋白質測定用キット。

【請求項 10】 更に抗C-反応性蛋白質抗体を含んでなる、請求項 9 記載のキット

【請求項 11】 請求項 1 ～ 6 の何れかに記載された酵素を用いることを特徴とす

る、C-反応性蛋白質の測定方法。

【請求項 1 2】更に抗C-反応性蛋白質抗体を組み合わせて用いる、請求項 1 1 記載の測定方法。

【請求項 1 3】C-反応性蛋白質を含む試料と、請求項 1 ～ 6 の何れかに記載された酵素と、抗C-反応性蛋白質抗体とを接触させた後、当該酵素の活性を測定し、得られた酵素活性値に基づいて試料中のC-反応性蛋白質量を求めることを特徴とする、C-反応性蛋白質の測定方法。

【請求項 1 4】特定位置にペプチドが挿入または置換されたグルコース-6-リン酸脱水素酵素。

【請求項 1 5】特定位置が、6 個以上のアミノ酸残基を有するペプチドの挿入または置換によっても、グルコース-6-リン酸脱水素酵素活性を保持できる位置である、請求項 1 4 記載の酵素。

【請求項 1 6】特定位置が、挿入または置換されたペプチドに該ペプチドと結合能を有する物質が結合すると、グルコース-6-リン酸脱水素酵素活性が変化する位置である、請求項 1 4 記載の酵素。

【請求項 1 7】特定位置が配列番号 6 で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列のLeu 3 0 5 - Asp 3 1 0 位、Glu 3 6 2 位及びC末端から選ばれる任意の位置である、請求項 1 4 記載の酵素。

【請求項 1 8】ペプチドがC-反応性蛋白質のアミノ酸配列から選ばれたものである、請求項 1 4 に記載の酵素。

【請求項 1 9】ペプチドが該ペプチドに特異的に結合する物質を有するものである、請求項 1 4 に記載の酵素。

【請求項 2 0】請求項 1 4 ～ 1 9 に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用試薬。

【請求項 2 1】請求項 1 4 ～ 1 9 に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用キット。

【請求項 2 2】請求項 1 4 ～ 1 9 に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドを含有する物質の測定方法。

【請求項 2 3】請求項 1 4 ～ 1 9 に記載された酵素と、該酵素に挿入または置換

されたペプチドと結合能を有する物質とを組み合わせ用いる、当該ペプチドを含有する物質の測定方法。

【請求項 2 4】請求項 1 4 ～ 1 9 に記載された酵素と、該酵素に挿入または置換されたペプチドを含有する物質を含む試料と、当該ペプチドに結合能を有する物質とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドを含有する物質の量を求めることを特徴とする、当該ペプチドを含有する物質の測定方法。

【請求項 2 5】請求項 1 4 ～ 1 9 に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用試薬。

【請求項 2 6】請求項 1 4 ～ 1 9 に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用キット。

【請求項 2 7】請求項 1 4 ～ 1 9 に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定方法。

【請求項 2 8】請求項 1 4 ～ 1 9 に記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質を含む試料とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドと結合能を有する物質の量を求めることを特徴とする、当該ペプチドと結合能を有する物質の測定方法。

【請求項 2 9】配列番号 6 で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列の Leu 3 0 5 - Asp 3 1 0 位、Glu 3 6 2 位及び C 末端から選ばれる任意の位置に、外来ペプチドが置換又は挿入されたアミノ酸配列を有するハイブリッド酵素をコードする遺伝子。

【請求項 3 0】請求項 2 9 記載のハイブリッド酵素遺伝子をベクター DNA に挿入したことを特徴とする新規な組み換え DNA。

【請求項 3 1】請求項 3 0 記載の組み換え DNA を含む形質転換体又は形質導入体。

【請求項 3 2】請求項 3 1 記載の形質転換体又は形質導入体を培養し、グルコース-6-リン酸脱水素酵素の酵素活性を有し、且つグルコース-6-リン酸脱水素酵素に置換又は挿入されたアミノ酸配列に、当該アミノ酸配列に結合能を有する物質

が結合すると、グルコース-6-リン酸脱水素酵素活性が変化する性質を有する蛋白質を回収することを特徴とする、上記性質を有する蛋白質の製造方法。

【請求項 3 3】 配列番号 6 で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列の Leu 3 0 5 - Asp 3 1 0 位、Glu 3 6 2 位及び C 末端から選ばれる任意の位置に、制限酵素により開環され得るアミノ酸配列が挿入あるいは置換されたアミノ酸配列を有する、ハイブリッド酵素をコードする遺伝子。

【請求項 3 4】 請求項 3 3 記載のハイブリッド酵素遺伝子をベクター DNA に挿入したことを特徴とする新規な組み換え DNA。

【請求項 3 5】 特定位置に、配列番号 1 に示したアミノ酸配列から選ばれたペプチドが挿入または置換された  $\beta$ -ガラクトシダーゼ。

【請求項 3 6】 特定位置が配列番号 3 0 で示される  $\beta$ -ガラクトシダーゼのアミノ酸配列の Ile280/Asp281、Val796/Ser797 の何れかから選択された部位である請求項 3 5 記載の  $\beta$ -ガラクトシダーゼ。

【請求項 3 7】 請求項 3 6 記載のハイブリッド酵素をコードする遺伝子。

【請求項 3 8】 請求項 3 7 記載のハイブリッド酵素遺伝子をベクター DNA に挿入したことを特徴とする新規な組み換え DNA。

【請求項 3 9】 請求項 3 8 記載の組み換え DNA を含む形質転換体または形質導入体。

【請求項 4 0】 請求項 3 9 記載の形質転換体または形質導入体を培養し、 $\beta$ -ガラクトシダーゼの酵素活性を表し、且つ  $\beta$ -ガラクトシダーゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、 $\beta$ -ガラクトシダーゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法。

【請求項 4 1】 特定位置に、配列番号 1 に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたアルカリフォスファターゼ。

【請求項 4 2】 特定位置が配列番号 3 1 で示されるアルカリホスファターゼのアミノ酸配列の Lys167/168Cys、Cys168/169Tyr、Glu407/408Asp、Lys91/93Thr、Tyr169/177Lys の何れかから選択された部位である請求項 4 1 記載のアルカリフォスファターゼ。

【請求項 4 3】請求項 4 2 記載のハイブリッド酵素をコードする遺伝子。

【請求項 4 4】請求項 4 3 記載のハイブリッド酵素遺伝子をベクター DNA に挿入したことを特徴とする新規な組み換え DNA。

【請求項 4 5】請求項 4 4 記載の組み換え DNA を含む形質転換体または形質導入体。

【請求項 4 6】請求項 4 5 記載の形質転換体または形質導入体を培養し、アルカリフォスファターゼの酵素活性を表し、且つアルカリフォスファターゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、アルカリフォスファターゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】

本発明はハイブリッド酵素およびそれを用いた生体試料成分分析測定系に関し、更に詳しくはC-反応性蛋白質（以下CRPと略記する）のアミノ酸配列の一部を含むハイブリッド酵素および該ハイブリッド酵素を用いたCRP測定系、特定位置に外来ペプチドを含有するハイブリッド型グルコース-6-リン酸脱水素酵素(G6PDH)及び該ハイブリッド酵素を用いた各種外来ペプチド測定系に関するものである。

【0002】

【従来の技術】

抗原抗体反応を利用した生体試料成分分析測定系としては、免疫比濁法、ラテックス凝集法等のホモジニアス測定系と、HPLC法、電気泳動法、放射線免疫測定法、酵素免疫測定法（以下、EIA法）等のヘテロジニアス測定系との2つに大別される。いずれの方法においても現在ではほとんど自動化された機器を用いて測定されているが、ヘテロジニアス測定系では固相を用いることから高分子物質測定であれ、低分子物質測定であれ特殊な機器が必須であり、汎用の自動分析機には適用できない。また、ホモジニアス測定系においても、免疫比濁法、ラテックス凝集法以外は特殊な機器が用いられ汎用の自動分析機には適用できない。し

かし、現在汎用の自動分析機に応用できるホモジニアスな免疫学的測定系として、酵素を用いた E M I T 法 (IMMUNOASSAYS R. M. Nakamura et. al Alan R. Liss, Inc., New York) が確立され、実用化されている。本測定系は、測定対象物質で化学修飾した酵素を用い、これに該測定対象物に対する抗体が結合すると酵素活性が阻害され低下する事を利用して、測定対象物の量を測定する方法であるが、測定対象物がハプテンなどのように低分子のものに限られるという問題点を有していた。そこで最近では、酵素を使用して高分子物質の測定を可能にするホモジニアスな免疫学的測定系として、遺伝子組み換えにより酵素中に外来ペプチドを挿入してハイブリッド酵素を生成しこれを用いたホモジニアス測定系が考案されている。即ち、当該ハイブリッド酵素中の外来ペプチド部分に抗体等が結合する際に酵素活性が変化することを利用して、そのペプチドあるいはそのペプチドをアミノ酸配列中に含む物質又はそのペプチドと結合能を有する物質の量を測定する方法であり、この方法で用いられる酵素としてはアルカリホスファターゼ (特表平8-507686号) や  $\beta$ -ガラクトシダーゼ (FEBS Letters434(1998)23-27、FEBS Letters438(1998)267-271) 等が知られている。しかしながらこれらの方法に於いては、外来ペプチドが挿入される位置により、当該ハイブリッド酵素が酵素活性を有さない場合、あるいは酵素活性があったとしても結合能を有する物質が該ハイブリッド酵素に結合したときに酵素活性が変化しない場合があること等も知られている。

一方、CRP は、肺炎球菌の莢膜の C 多糖体と反応する血清中の  $\beta$  グロブリンであるが、急性期反応蛋白であり組織破壊を伴うような炎症性疾患時等に増加することが知られており、CRP の測定は、組織中の炎症の早期発見、病気の診断等に有用である。この CRP の測定は、従来、免疫比濁法、ラテックス凝集法、酵素免疫法、放射性免疫法等により行われている。これらのうち、免疫比濁法、ラテックス凝集法は、汎用自動分析装置に適応可能な方法であるが、どちらも濁度変化を検出するものであるため、完全なホモジニアス測定系とは言えず、測定精度の面において問題がある。従って、測定精度が良い完全なホモジニアス系による汎用自動分析装置に適応可能な CRP の微量測定法の開発が望まれている。

【0003】



## 【発明が解決すべき課題】

上記した如き状況に鑑み、本発明が解決しようとする課題の1つは、ホモジニアスな比色法で試料中の微量なCRP測定を可能とする方法を提供することにある。もう一つの本発明が解決しようとする課題は、高分子物質をホモジニアスな系で測定するための方法を提供することにある。

## 【0004】

## 【課題を解決するための手段】

本発明者らは上記課題を解決すべく研究を重ねた結果、上述したハイブリッド酵素を用いれば、高分子の測定対象物を、ホモジニアスな系で測定することが可能であり、また、その検出は比色法で行うことが可能であることから、この方法をCRPの測定に応用すれば、汎用自動分析装置に適応可能なCRPの新たな微量分析法が開発し得ると考え、更に研究を重ねて、ある種の酵素のある位置にCRP等の被分析物質の一部を含有するハイブリッド酵素を用いることにより上記の課題が解決されることを見出し、本発明に到達したものである。

即ち、本発明は、特定の酵素中にCRP由来のペプチドを挿入したハイブリッド酵素を用いて、ホモジニアスな比色法で試料中の微量なCRP測定を可能とする方法を提供する為のハイブリッド酵素として、(1)配列番号1に示したアミノ酸配列の一部を含むペプチドにより一部が置換されるか、又は当該ペプチドが挿入されたアミノ酸配列を有するハイブリッド酵素であって、当該ハイブリッド酵素が、前記ペプチドが置換又は挿入される前と同様の酵素活性を有し且つ置換又は挿入されたペプチド部分に当該ペプチド対して結合能を有する物質が結合すると当該酵素活性が変化する性質を有するハイブリッド酵素、(2)ペプチドが、配列番号1に示したアミノ酸配列のうちから選ばれた少なくとも6個以上の連続したアミノ酸配列を含むものである(1)記載のハイブリッド酵素、(3)ペプチドが、CRPと結合能を有する物質と結合可能な性質を有するものである(2)記載のハイブリッド酵素、(4)ペプチドのアミノ酸配列が配列番号2～5の何れかの配列から選ばれた少なくとも6個以上のアミノ酸配列を含むものである(1)記載のハイブリッド酵素、(5)酵素が、グルコース-6-リン酸脱水素酵素(以下G6PDHと略記する場合がある)、 $\beta$ -ガラクトシダーゼ又はアルカリホ

スファターゼである（１）記載のハイブリッド酵素、（６）ペプチドと結合能を有する物質が抗体である（１）記載のハイブリッド酵素が、有効であることを見出したものである。そして上記のハイブリッド酵素を用いたホモジニアスな比色法で試料中の微量なＣＲＰ測定方法として、（７）（１）～（６）の何れかに記載された酵素を含んでなるＣＲＰ測定用試薬、（８）更に抗ＣＲＰ抗体を含んでなる（７）記載の試薬、（９）（１）～（６）の何れかに記載された酵素を含む試薬を含んでなるＣＲＰ測定用キット、（１０）更に抗ＣＲＰ抗体を含んでなる、請求項９記載のキット、（１１）（１）～（６）の何れかに記載された酵素を用いることを特徴とするＣＲＰの測定方法、（１２）更に抗ＣＲＰ抗体を組み合わせ用いる（１１）記載の測定方法、（１３）ＣＲＰを含む試料と、（１）～（６）の何れかに記載された酵素と、抗ＣＲＰ抗体とを接触させた後、当該酵素の活性を測定し、得られた酵素活性値に基づいて試料中のＣＲＰ量を求めることを特徴とする、ＣＲＰの測定方法、を提供するものである。

また本発明では高分子物質をホモジニアスな系で測定するために、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、Ｇ６ＰＤＨ含有ハイブリッド酵素として、（１４）特定位置にペプチドが挿入または置換されたＧ６ＰＤＨ、（１５）特定位置が、６個以上のアミノ酸残基を有するペプチドの挿入または置換によっても、Ｇ６ＰＤＨ活性を保持できる位置である（１４）記載の酵素、（１６）特定位置が、挿入または置換されたペプチドに該ペプチドと結合能を有する物質が結合すると、Ｇ６ＰＤＨ活性が変化する位置である（１４）記載の酵素、（１７）特定位置が配列番号６で示されるＧ６ＰＤＨのアミノ酸配列のLeu 3 0 5 - Asp 3 1 0 位、Glu 3 6 2 位及びＣ末端から選ばれる任意の位置である（１４）記載の酵素、（１８）ペプチドがＣＲＰのアミノ酸配列から選ばれたものである（１４）記載の酵素、（１９）ペプチドが該ペプチドに特異的に結合する物質を有するのである（１４）記載の酵素、が有効であることを見出したものである。更に（２０）（１４）～（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用試薬、（２１）（１４）～（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測

定用キット、(22)(14)～(19)に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドを含有する物質の測定方法、(23)(14)～(19)に記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質とを組み合わせ用いる、当該ペプチドを含有する物質の測定方法、(24)(14)～(19)に記載された酵素と、該酵素に挿入または置換されたペプチドを含有する物質を含む試料と、当該ペプチドに結合能を有する物質とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドを含有する物質の量を求めることを特徴とする、当該ペプチドを含有する物質の測定方法、(25)(14)～(19)に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用試薬(26)(14)～(19)に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用キット、(27)(14)～(19)に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定方法、(28)(14)～(19)に記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質を含む試料とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドと結合能を有する物質の量を求めることを特徴とする、当該ペプチドと結合能を有する物質の測定方法を提供するものである。

また本発明ではある物質或いはこれに結合能を有する物質を検出するための試薬を製造するために、(29)配列番号6で示されるG6PDHのアミノ酸配列のLeu305-Asp310位、Glu362位及びC末端から選ばれる任意の位置に、外来ペプチドが置換又は挿入されたアミノ酸配列を有するハイブリッド酵素をコードする遺伝子が有用であることを見出したものである。更に本発明は、(30)(29)記載のハイブリッド酵素遺伝子をベクターDNAに挿入したことを特徴とする新規な組み換えDNA、(31)(30)記載の組み換えDNAを含む形質転換体又は形質導入体、(32)(31)記載の形質転換体又は形質導入体を培養し、G6PDHの酵素活性を有し、且つG6PDHに置換又は挿入されたアミノ酸配列に、当該アミノ酸配列に結合能を有する物質が結合すると、G6PDH活性が変化する

性質を有する蛋白質を回収することを特徴とする、上記性質を有する蛋白質の製造方法、(33) 配列番号6で示されるG6PDHのアミノ酸配列のLeu305-Asp310位、Glu362位及びC末端から選ばれる任意の位置に、制限酵素により開環され得るアミノ酸配列が挿入あるいは置換されたアミノ酸配列を有する、ハイブリッド酵素をコードする遺伝子、(34) (33) 記載のハイブリッド酵素遺伝子をベクターDNAに挿入したことを特徴とする新規な組み換えDNA、を提供するものである。

さらには、(35) 特定位置に、配列番号1に示したアミノ酸配列から選ばれたペプチドが挿入または置換された $\beta$ -ガラクトシダーゼ、(36) 特定位置が配列番号30で示される $\beta$ -ガラクトシダーゼのアミノ酸配列のIle280/Asp281、Val796/Ser797の何れかから選択された部位である(35) 記載の $\beta$ -ガラクトシダーゼ、(37) (36) 記載のハイブリッド酵素をコードする遺伝子、(38) (37) 記載のハイブリッド酵素遺伝子をベクターDNAに挿入したことを特徴とする新規な組み換えDNA、(39) (38) 記載の組み換えDNAを含む形質転換体または形質導入体、(40) (39) 記載の形質転換体または形質導入体を培養し、 $\beta$ -ガラクトシダーゼの酵素活性を表し、且つ $\beta$ -ガラクトシダーゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、 $\beta$ -ガラクトシダーゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法を提供するものである。

さらにまた、(41) 特定位置に、配列番号1に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたアルカリフォスファターゼ、(42) 特定位置が配列番号31で示されるアルカリホスファターゼのアミノ酸配列のLys167/168Cys、Cys168/169Tyr、Glu407/408Asp、Lys91/93Thr、Tyr169/177Lysの何れかから選択された部位である(41) 記載のアルカリフォスファターゼ、(43) (42) 記載のハイブリッド酵素をコードする遺伝子、(44) (43) 記載のハイブリッド酵素遺伝子をベクターDNAに挿入したことを特徴とする新規な組み換えDNA、(45) (44) 記載の組み換えDNAを含む形質転換体または形質導入体、(46) (45) 記載の形質転換体または形質導入体を培養

し、アルカリフォスファターゼの酵素活性を表し、且つアルカリフォスファターゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、アルカリフォスファターゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法を提供するものである。

【 0 0 0 5 】

#### 【発明の実施形態】

本発明のハイブリッド酵素に用いるオリジン酵素としては、一般的に用いられている酵素であればどのようなものを用いてもよいが、アデノシンデアミナーゼ；アルカリ性ホスファターゼ； $\alpha$ -アミラーゼ；細菌ルシフェラーゼ； $\beta$ -ガラクトシダーゼ； $\beta$ -ガラクトシダーゼフラグメント； $\beta$ -ラクタマーゼ；カルボニックアンヒドラーゼ；カタラーゼ；ホタルルシフェラーゼ；グルコースオキシダーゼ；グルコース-6-リン酸脱水素酵素；グルコシダーゼ；ヘキソキナーゼ；西洋ワサビペルオキシダーゼ；インペルターゼ；イソクエン酸デヒドロゲナーゼ；リゾチーム；リンゴ酸デヒドロゲナーゼ；マイクロペルオキシダーゼ；6-ホスホフラクターゼ；及びキサンチンオキシターゼなどが挙げられる。

さらに、アミノ酸配列や遺伝子配列が明らかであるか、明らかにし得るものであり、外来ペプチドにより一部が置換されるか外来ペプチドを挿入し、当該ペプチドに対して結合能を有する物質が結合すると当該酵素活性が変化するものはすべて使用可能である。中でも、酵素活性が高いもの、安定性がよいもの、比色法で測定できるもの等が好ましく、例えば、G 6 P D H、 $\beta$ -ガラクトシダーゼ、アルカリホスファターゼ等が挙げられる。また、これらの酵素の遺伝子は、通常一般的に行われるゲノムからのクローニング方法等で入手可能であり、もちろん既にクローニングされた遺伝子や合成DNA等を使用することもできる。また、これらの酵素の由来はいかなるものであってもよく、そのアミノ酸配列については本来の酵素活性を有するものであれば1もしくは複数のアミノ酸が欠失、置換あるいは付加された配列であってもよい。

本発明のハイブリッド酵素を作製するために用いられる外来ペプチドとしては、そのペプチドに結合能を有する抗体やレセプター等の物質が存在するものであ

ればどのようなものを用いてもよく、一般に免疫学的測定系で測定されている、例えば、生体成分であるCRP、IgG、IgA、IgM、C 3、C 4、 $\beta$  2ミクログロブリン等、あるいは、 $\alpha$ -フェトプロテイン、CA19-9、前立腺特異抗原(PSA)、癌胎児性抗原(CEA)等の各種ガンマーカー、あるいは、インシュリン、ヒト絨毛性ゴナドトロピン(hCG)、アルブミン、ストレプトトリジンO (S L O)、プロラクチン、甲状腺刺激ホルモン(TSH)等の各種ホルモン、あるいは、B型肝炎ウイルス(HBV)、C型肝炎ウイルス(HCV)、ヒト免疫不全ウイルス(HIV)、成人T細胞白血病ウイルス(HPV)等の各種ウイルス等がその供与体として挙げられる。

本発明の組み換えDNAに用いるベクターとしては、プラスミドベクター及びファージベクター等の原核細胞および／または真核細胞の各種宿主内で複製保持または自己増殖できるものであれば特に限定されないが、例えば当業界において入手可能な、pBR322、pBR325、pUC12、pUC13、pBluescript等の大腸菌由来プラスミド、pSH19、pSH15等の酵母由来プラスミド、pUB110、pTP5、pC194等の枯草菌由来プラスミド等が挙げられる。また、ファージベクターとしては $\lambda$ ファージなどのバクテリオファージ、更には、レトロウイルス、ワクシニアウイルス、核多角体ウイルスなどの動物や昆虫のウイルス等が挙げられる。

本発明のハイブリッド酵素の生産に用いる宿主細胞としては、細菌（例えば大腸菌など）、酵母（例えばサッカロマイセス属など）、動物細胞（例えばチャイニーズハムスター細胞CHOなど）、昆虫細胞（例えばB m N 4 など）が挙げられる。

本発明のハイブリッド酵素を生産するための、組み換えDNAの構築や発現及び精製は、自体公知の方法、例えばMolecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載されている方法に従えばよい。

また、発現ベクターとしては、原核細胞および／または真核細胞の各種宿主内で複製保持または自己増殖できるものであって、原核細胞および／または真核細胞の各種宿主細胞中でハイブリッド酵素をコードする遺伝子を発現させる機能、即ち、目的のハイブリッド酵素を生産させる機能を有するものであれば特に限定されないが、例えば当業界において入手可能ものとして、宿主細胞が大腸菌の場合

合は、pBR322、pUC12、pUC13、pTrcHis、pTrc99A、pMAL-c2、もしくはその人工的修飾物（該ベクターを適当な制限酵素で処理して得られるDNAフラグメント）等が、宿主細胞が酵母の場合は、プラスミドpRS403、pRS404、pRS413、pRS414、pYES2等が、宿主細胞が動物細胞の場合は、プラスミドpRSVneo ATCC37224、pSV2dhfr ATCC37145、pdBPV-MMTneo ATCC37224、pSV2neo ATCC37149等が、宿主細胞が昆虫細胞の場合は、オートグラフィカル カリホルニカ (*Autographica californica*) 核多角体ウイルス (AcNPV)、ボンビックス モリ (*Bombyx mori*) 核多角体ウイルス (BmNPV) 等が好ましい。

本発明のハイブリッド酵素の作製について、G 6 P D Hをオリジン酵素として、C R P由来ペプチドを外来ペプチドとして、また、大腸菌を宿主細胞として使用した場合を例にとって説明する。

G 6 P D H遺伝子は、例えば以下に示す方法で得ることができる。即ち、先ずMolecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載の常法に従って*Leuconostoc mesenteroides*の培養物より遠心分離で集菌し、ゲノムDNAを抽出する。尚、以下では特に記載しない限り、Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載された、一般的に行われる周知の技術に従って操作を行う。上記ゲノムDNAを鋳型とし、R.Levyらの文献(J.Biological Chemistry, Vol.266, p13028-, 1991)に示されたG 6 P D H遺伝子の配列より、G 6 P D H遺伝子のN-及びC-末端配列を含むか、あるいはこれより上流及び下流域の配列を有するオリゴヌクレオチドプライマーを加え、DNAサーマルサイクラー（パーキンエルマー社）を用いてポリメラーゼ連鎖反応を行い、G 6 P D H遺伝子を含むDNA断片を特異的に増幅させる。得られたDNA断片を常法に従いベクターDNAに組み込むことで、G 6 P D HをコードするDNAを含む、組み換えDNAを得ることができる。

さらに、外来ペプチドをG 6 P D H遺伝子に挿入あるいは置換の形で連結するには、いかなる方法でもよく、周知技術である、例えば、「ラボマニュアル遺伝子工学」（村松正貫 編、第3版、p219～230、丸善）に記載のエキソヌクレアーゼを用いた欠失変異体作製方法やKunkel法、カセット法、PCRを用いる方法の

ような人工変異導入法、あるいは「DNAクローニング1」(D.M.GLOVERら編、第2版、p197~228、Takara社)に記載のホスホロチオエート法、ギャップ二本鎖DNA法、MHTプロトコール等を組み合わせて行うことができる。得られたハイブリッド酵素をコードするDNAを常法に従いベクターDNAに組み込むことで、ハイブリッド酵素をコードするDNAを含む組み換えDNAを得ることができる。ここでは、プラスミドに組み込まれたG6PDH遺伝子中に制限酵素部位を作製し、外来ペプチドを挿入あるいは置換の形で連結する方法について説明する。

即ちG6PDH遺伝子を鋳型とし、ペプチドを挿入したい部分の両側のアミノ酸配列をコードする塩基配列の5' - 側に制限酵素、例えばBamHIにより切断され得る部位(BamHI部位)を付加したプライマーと、これより上流および下流部分をコードする塩基配列のプライマーを組み合わせて、PCRを行い、BamHI部位が付加したDNA断片を増幅する。次に、得られた2種類の断片をベクター上で連結することにより、BamHI部位が挿入されたG6PDH遺伝子が構築できる。制限酵素部位を制限酵素で切断することにより、切断部位と相補的な配列を両端に持つ任意のDNA断片を挿入することができる。

また、ペプチドを置換挿入するために任意のアミノ酸配列を取り除く場合は、取り除く配列の両側について上記と同様の操作を行うことにより、外来ペプチドが置換の形で連結されたG6PDH遺伝子を得ることが出来る。

組換えDNAの構築の際には、他の蛋白やペプチドとハイブリッド酵素との融合蛋白として分泌生産させることもでき、さらに、融合蛋白として分泌生産させた後に、適当なプロテアーゼや化学処理による切断を行い、ハイブリッド酵素を得ることもできる。融合させる蛋白としては、例えば、マルトース結合蛋白やグルタチオンS-トランスフェラーゼなどが、融合させるペプチドとしてはヒスチジンタグ、FLAGタグなどが挙げられる。

組換えDNA構築の基礎となるベクターとしては、例えば、pBR322(J.G.Sutcliffe, Cold Spring Harbor Symposium, 43, 77, 1979)、pUC18/19(C. Yanisch-Perron, et. al, Gene 33, p102-119, 1985)、pBluescript IISK+ (STRATEGENE社)、pMAL-C2 (NEW England Biolabs社)、pTrc99A (アマシャム ファルマシア社)、p



KK223-3 (アマシャム ファルマシア社) pET-11 (STRATEGENE社) 等のプラスミドベクター、 $\lambda$  ENBL3 (STRATEGENE社)、 $\lambda$  DASHII (フナコシ社) 等のバクテリオファージベクターが挙げられる。

組換えDNAに用いるプロモーターとしては、大腸菌で機能するものであればいずれでもよく、例えば、lacプロモーター、trpプロモーター、T7プロモーターおよびこれらの誘導体などが挙げられる。また、組換えDNAは、大腸菌で機能するリボソーム結合配列等の開始シグナルや、ターミネーター等を含んでいてもよい。さらに、アンピシリン耐性遺伝子、テトラサイクリン耐性遺伝子などの選択マーカー遺伝子などを含んでいてもよい。

【 0 0 0 6 】

このようにして構築した組換えDNAを用いて大腸菌を形質転換または形質導入し、形質転換体または形質導入体を作製する。大腸菌としては、M103、JA221、HB101、C600、XL1-Blue、JM109などが挙げられる。

組換えDNAを大腸菌へ形質転換または形質導入する方法としては、例えば、Cohenらの方法 (Proc.Natl.Acad.Sci.U.S.A.,69,p2110,1972) ,Hanahanらの方法 (J.Mol.Biol.,166,p557,1983) などが挙げられる。なお、組み換えDNAを有する形質転換体または形質導入体から当該組み換えDNAを得るには、例えばアルカリミニプレップ法等の常法により行えばよい。

本発明のハイブリッド酵素は、上記のごとく調製される組換えDNAの形質転換体または形質導入体を培養することによって製造することができる。使用する培地としては、例えばLuria-Bertani培地 (Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)、2×YT培地(Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)、M9培地(J.Miller.Exp.Mol.Genet.,Cold Spring Harbor Laboratory,New York,p431,1972)などが挙げられ、培地のpHは5～8であることが望ましい。培養は、通常14～42℃、好ましくは28～39℃で3～24時間行うことができ、必要に応じて通気や攪はんを加えてもよい。また、必要により、isopropyl- $\beta$ -D-1-thiogalactopyranoside等の発現誘導剤や、アンピシリンやクロラムフェニコール等の抗生物質等を添加してもよい。

本発明のハイブリッド酵素は、上記培養により得られる培養物より以下のようにして取得できる。即ち、ハイブリッド酵素が、培養物のうち培養液中に存在する場合は、得られた培養物をろ過または遠心分離等の常法により、ハイブリッド酵素を含有する培養ろ液または培養上清を得る。一方、ハイブリッド酵素が、培養された形質転換体または形質導入体のペリプラズムまたは細胞内に存在する場合は、培養物をろ過または遠心分離等の常法に付して菌体を集め、適当な緩衝液に懸濁し、例えば超音波やリゾチーム及び凍結融解等の常法で細胞を破壊した後、ろ過または遠心分離等の常法でハイブリッド酵素を含有する粗抽出液を得る。

このようにして得られた本発明のハイブリッド酵素を含有する培養ろ液、培養上清あるいは粗抽出液からハイブリッド酵素を分離、精製するには、自体公知の分離、精製法を適切に組み合わせて実施すればよい。これらの公知の分離、精製法としては、塩析や溶媒沈殿法などの溶解度の差を利用する方法、透析法、限外濾過法、ゲル濾過法、あるいはSDS-ポリアクリルアミドゲル電気泳動法などの主として分子量の差を利用する方法、イオン交換クロマトグラフィーなどの荷電の差を利用する方法、疎水クロマトグラフィーなどの疎水性の差を利用する方法、等電点電気泳動法などの等電点の差を利用する方法、アフィニティークロマトグラフィーなどの特異的親和性を利用する方法などが挙げられる。

得られたハイブリッド酵素の酵素活性に対する抗CRP抗体の効果は、例えば以下のようにして調べることができる。即ち、抗CRP抗体の非存在及び存在下でハイブリッド酵素の酵素活性をそれぞれ測定し、抗CRP抗体結合による活性の変動を調べる。各ハイブリッド酵素液を1%牛アルブミン、3mM塩化マグネシウム、150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8)で約1 U/mlに希釈したもの6  $\mu$ lに、3mM塩化マグネシウムと150mM塩化ナトリウムを含有する100mM

Tris/HCl緩衝液(pH7.8; 以下、緩衝液Aと省略する)、または抗CRPヤギ抗体を緩衝液Aで100倍希釈した抗体液を150  $\mu$ l添加し、37℃で5分間反応後、10mM グルコース-6-リン酸(G6P)、6mM ニコチンアミドアデニンジヌクレオチド(NAD)を含有する緩衝液A 75  $\mu$ lを添加し、37℃で5分間反応させ、波長340nmにおける5分間の吸光度変化をG6PDH活性として求める。

【 0 0 0 7 】

酵素中に挿入する外来ペプチドは、該ペプチドに結合能を有する物質が結合できる構造を維持しており、該ペプチドの挿入によっても酵素活性が失活しないものであればよいが、Antibodies A Laboratory Manual (Ed Harlow et.al, p76-, Cold spring Harbor Laboratory) 等に記載のように、挿入されるペプチドが抗原性を維持するためには少なくとも6個以上の連続するアミノ酸残基を有する構造である必要がある。また、挿入する外来ペプチドがCRP由来のペプチドである場合には、配列番号1で示されるCRPの全アミノ酸配列の全部或いは一部が挙げられ、さらには、そのうちの6～50個の連続したアミノ酸配列を含むもの、例えばGln(1)～Asp(16)、Glu(14)～Ala(24)、Leu(22)～Ser(45)、Thr(41)～Asn(61)、Arg(47)～Ile(63)、Lys(114)～Lys(121)、Glu(130)～Glu(138)、Ile(134)～Gly(148)、Gln(137)～Leu(152)、Glu(147)～Leu(152)、Asp(3)～Ser(18)、Leu(152)～Val(165)、Val(165)～Gly(178)、Leu(121)～Ser(132)、Arg(188)～Glu(197)から選ばれる連続したアミノ酸配列が好ましい。中でも配列番号2: Asp(3)～Ser(18)、配列番号3: Leu(152)～Gly(178)、配列番号4: Leu(121)～Ser(132)、配列番号5: Arg(188)～Glu(197)に示される配列から選ばれる、少なくとも6個以上の連続したアミノ酸配列を含むものがより好ましい。なお、酵素中に挿入あるいは置換されるペプチド部分には、上記ハイブリッド酵素作成過程で入ることのある制限酵素部位等、目的のペプチド以外の配列が含まれることがあってもよい。

本発明におけるハイブリッド酵素のオリジン酵素に外来ペプチドを挿入する位置は、外来ペプチドの挿入によっても該酵素活性が保持され、挿入された外来ペプチドに該ペプチドと結合能を有する物質が結合すると酵素活性が変化する位置であればよく、酵素表面に露出している部位、活性化に影響し得る部位が候補として挙げられる。オリジン酵素としてのG6PDHに外来ペプチドを挿入する好ましい位置についてCRP由来ペプチドを用いて検討した。即ち、そのN末端、

32/33 (32番目と33番目の間の意、以下同様)、37/38、48/49、66/67、87/88、226/227、296/297、305~310、362/363、409/410、C末端について外来ペプチドの挿入を試みたが表1に示すように、ペプチド挿入時に酵素活性が残存し、抗CRP抗体反応時により酵素活性が変動するCRP測定に有効な部位は、305/306、306/307、308/309、309/310、362/363及びC末端であることが判明した。

【0008】

【表1】

| 挿入部位          | ペプチド挿入時の活性 | 抗体反応時の酵素活性変動 |
|---------------|------------|--------------|
| Lys32/Lys33   | —          | —            |
| Gln37/Lys38   | —          | —            |
| Gln48/Ala49   | +          | —            |
| Phe66/Thr67   | +          | —            |
| Val87/Thr88   | —          | —            |
| Gly226/Tyr227 | —          | —            |
| Ala296/Asp297 | —          | —            |
| Leu305/Asp306 | +          | +            |
| Asp306/Val307 | +          | +            |
| Pro308/Ala309 | +          | +            |
| Ala309/Asp310 | +          | +            |
| Glu329/Gly310 | —          | —            |
| Glu362/Gln363 | +          | +            |
| Lys409/Lys410 | —          | —            |
| C-末           | +          | +            |

酵素のアミノ酸配列の一部が外来ペプチドにより置換されるという意味は、酵素の特定部位のアミノ酸残基またはアミノ酸配列が外来ペプチドのアミノ酸配列に置き換わることをいう。この場合、置換後にもオリジン酵素の酵素活性が保持され、置換された外来ペプチドに該ペプチドと結合能を有する物質が結合すると該酵素活性が変化するようであれば、取り除かれるアミノ酸残基或いはアミノ酸配列に対して置換挿入されるペプチドのアミノ酸の残基数は多くても少なくともよいが、挿入されるペプチドのアミノ酸残基数と同じ程度が好ましい。また、置換挿入されるペプチドのアミノ酸残基数は、上記の酵素中に挿入する外来ペプチドのそれと同じ程度が好ましい。さらに、外来ペプチドが置換される位置としては、置換後にもオリジン酵素の酵素活性が保持され、置換された外来ペプチドに該ペプチドと結合能を有する物質が結合すると該酵素活性が変化するようであ

ばよく、上記に示したハイブリッド酵素のオリジン酵素に外来ペプチドを挿入し得る位置に準じて選択される。

【 0 0 0 9 】

本発明では、上記のようにして得られたハイブリッド酵素を定性または定量分析に使用する。

本発明のハイブリッド酵素は挿入あるいは置換された外来ペプチドに結合能を有する物質が該酵素に結合すると、該酵素活性がその結合量に応じて変化するものである。従って、本発明のハイブリッド酵素に該結合物質を含む試料を反応させ、酵素活性の変動を測定することにより該結合能を有する物質の存在及びその量を検出することができる。また、本発明のハイブリッド酵素と、該結合能を有する物質とを用いれば、ハイブリッド酵素に挿入あるいは置換された該ペプチドと該ペプチドを含有する物質の該結合能を有する物質への競合により、ハイブリッド酵素に結合する該結合能を有する物質の量が変化することを利用して、該ペプチドを含有する物質の存在またはその量を検出することができる。その方法は（１）被分析物質を含む試料と、本発明のハイブリッド酵素と、本発明のハイブリッド酵素に挿入あるいは置換された外来ペプチドに結合能を有する物質とを接触させ、反応混合物を形成するステップ；（２）前記反応混合物を、出発酵素に対する基質と接触させるステップ；及び（３）反応混合物中に存在する被分析物質の量に従い、ハイブリッド酵素の酵素活性の変化をモニターするステップを含む。ステップ（２）は、反応混合物を定常または平衡状態としてから実施することもできるし、またステップ（１）は順次または同時に実施することができる。

（１）において、該結合能を有する物質を含む試料と、ハイブリッド酵素を反応させる操作を行うことにより、該結合能を有する物質の存在及びその量の検出を行うことが出来る。

このように、本発明のハイブリッド酵素含有試薬を用いれば、抗ＣＲＰ抗体の存在または量を直接に測定することが出来、また本発明のハイブリッド酵素に更に抗ＣＲＰ抗体を含有する試薬を用いて、結合分子たる抗ＣＲＰ抗体への結合の競合によって抗原たるＣＲＰの存在または量を間接的に検出するアッセイを行うことができる。

尚、ハイブリッド酵素の酵素活性の測定は、オリジン酵素の活性測定法に準じて行えばよい。

また、高分子物質をホモジニアスな系で測定するための、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、G6PDH含有ハイブリッド酵素を作成するために用いられるG6PDHとしては、配列番号6に示したアミノ酸配列、或いは当該配列のうち、1もしくは複数のアミノ酸が欠失、置換あるいは付加された配列を有するものや、由来の異なるG 6 P D Hであっても、G 6 P D H活性を有するもの等が含まれる。また、外来ペプチドが挿入される位置については上記で示した位置が好ましいものとして挙げられる。

また、外来ペプチドとしては先に挙げた全てのものが挙げられ、このうちC R Pについては、上記のようなペプチドを用いることが好ましい。

また、G 6 P D H以外の酵素を用いて作成されるC R P由来のペプチドをその一部として含む外来ペプチドが挿入または置換されたハイブリッド酵素も上で述べたものと同様な方法により作成される。また、外来ペプチドが挿入または置換される位置についても同様にして適宜選択すればよい。尚、酵素として $\beta$ -ガラクトシダーゼを用いる場合は、FEBS Letters 434(1998)23-27、FEBS Letters 438(1998)267-271の記載に準じて、アルカリホスファターゼを用いる場合にはProc. Natl. Acad. Sci. USA 92(1995)に記載の方法に準じて選択してもよい。

このようにして得られた、外来ペプチドが挿入されたハイブリッド酵素を用いれば、当該外来ペプチドに結合能を有する物質の存在またはその量を直接に測定することができ、また、当該ハイブリッド酵素と当該外来ペプチドに対する抗体とを組み合わせ用いれば、当該外来ペプチドを含む高分子物質の存在又は量を間接的に検出するアッセイを行うことができる。尚、これらアッセイは、上で述べたC R P或いは抗C R P抗体の測定操作に準じて行えばよい。更に、ハイブリッド酵素を用いることによりホモジニアスな系で高分子物質を高感度に測定することが出来、この方法は汎用の自動分析装置に応用することもできる。

以下に本発明を更に詳しく説明するために実施例を挙げるが、本発明はこれに限定されるものではない。

【 0 0 1 0 】

## 【実施例】

## 実施例 1

## G6PDH遺伝子を含むプラスミドの構築

*Leuconostoc mesenteroides*をLACTOBACILLI MRS BROTH (DIFCO社) 5ml に接種して、26℃で16時間振とう培養し培養物を得た後、この培養物を4℃、6000rpmで10分間遠心分離することにより集菌して菌体を得た。1mM エチレンジアミン四酢酸(EDTA)を含有する10mMトリス(ヒドロキシメチル)アミノメタン(Tris/HCl) (以下、TEと省略する。)緩衝液に懸濁し、終濃度300u/mlのアクロモペプチダーゼを添加して37℃、2時間静置した後、終濃度0.5%のSDSと終濃度100 μg/mlのProteinase Kを添加して、さらに37℃、2時間静置して溶菌を行った。Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載の定法に従って、G6PDH (以下、G6PDHと省略する。)遺伝子の供与体として、*Leuconostoc mesenteroides*のゲノムDNAを抽出した。なお、以下では特に記載しない限り、Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載された、一般的に行われる周知の技術に従って操作を行った。次に、G6PDH遺伝子を得るために、以下の手順でポリメラーゼ連鎖反応(以下、PCRと省略する。)を行った。鋳型DNAとして上記ゲノムDNAを10ng、R.Levyらの文献(J.Biological Chemistry, Vol.266, p13028-, 1991)に示されたG6PDH遺伝子のN-及びC-末端配列を含む、各々配列番号7、8に記載のオリゴヌクレオチドプライマーを各0.1 nmol加え、DNAサーマルサイクラー(パーキン・エルマー社)を用い、94℃で30秒、65℃で30秒、72℃で4分のサイクルを25回繰り返して反応を行った。その結果、G6PDH遺伝子を含む約1.5 kbpのDNA断片が特異的に増幅された。得られたDNA断片をクローニングベクターpBluescript I I KS+ (Stratagene社)のEcoRV部位へ連結して、プラスミドpBSWGを構築した。

次いで、クローニングベクターpUC18をEcoRIとSalIで消化した後、その末端を平滑化したものに得られたDNA断片を連結し、G6PDH遺伝子の発現が可能なプラスミドでpUCGを構築した。さらに、Kunkel法に従い、配列番号9に示し

たオリゴヌクレオチドプライマーとMutan-K（宝酒造社）を使用して、G6PDH遺伝子のN-末端から750番目の塩基シトシンをチミンに変異させ、アミノ酸配列を変えることなく、N-末端以外には制限酵素Nco I認識配列を有さないG6PDH遺伝子を含む、プラスミドpBSMGを構築した。これを制限酵素NcoI及びPst Iで消化して、約1.5kbpのG6PDH遺伝子を回収し、プラスミドpUCGを制限酵素Nco I及びPst Iで消化した約2.7kbpのDNA断片と連結して、プラスミドpUCMGを構築した。

【0011】

## 実施例 2

G6PDHのPro308/Ala309間にヒトCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例1のプラスミドpUCMGを鋳型とし、配列番号7及び、Pro308上流のアンチセンス鎖配列の5' -側に制限酵素BamH I認識配列を付加した配列番号10に記載のオリゴヌクレオチドプライマーを用いてPCRを行い、下流部位に制限酵素BamH I認識配列が付加されたG6PDH遺伝子N末端からPro308までを含む約0.9kbpのDNA断片を得た。同様に、Ala309下流のセンス鎖配列の5' -側に制限酵素BamH I認識配列を付加した配列番号11に記載のオリゴヌクレオチドプライマーと、G6PDH遺伝子C-末端のアンチセンス鎖配列を含む配列番号12に記載のオリゴヌクレオチドプライマーを用いて、上流部位に制限酵素BamH I認識配列が付加された、G6PDH遺伝子のAla309からC末端を含む約0.6kbpのDNA断片を得た。N末端側断片を制限酵素BamH IとNco I、C末端側断片を、制限酵素BamH IとPst Iでそれぞれ消化した後、プラスミドpUCMGを制限酵素Nco I とPst I 部位で消化して得られる約2.7kbpのDNA断片と連結することで、G6PDH遺伝子のPro308/Ala309間にのみ制限酵素BamH I 配列を有する組み換え体 pUCMG308Bを構築した。これを制限酵素BamH I で開裂させ、配列番号2に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号13と配列番号14との組合せ)を連結しpUCMG308C1を、配列番号3に記載のアミノ酸配列の一部をコードするDNAを含み且つそれらが相補な合成ヌクレオチドである配列番号15と配列番号16との組合せ又は配列番号21と配列番号22との組合せを連結しpUCMG308C2及びpUCMG308C13を、



配列番号4に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号17と配列番号18との組合せ)を連結しpUCMG308C3を、配列番号5に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号19と配列番号20との組合せ)を連結しpUCMG308C5を、夫々構築した。尚、各合成ヌクレオチドは次のような相補構造から成っている。

## 【化1】

|   |        |
|---|--------|
| 5'-gatccgacatgtcgaggaagcttttgtgtttccaaagagtcggatacttccg -3'   | 配列番号13 |
| 3'- gctgtacagctccttccgaaaacacaaagggtttctcagcctatgaaggcctag-5' | 配列番号14 |
| 5'-gatccgtgctgtcaccagatgagattaacaccatctatcttgccgggg -3'       | 配列番号15 |
| 3'- gcacgacagtggtctactctaattgtggtagatagaaccgccccctag-5'       | 配列番号16 |
| 5'-gatccctgaagaagggatacactgtggggcagaagcaagcg -3'              | 配列番号17 |
| 3'- ggacttcttcctatgtgacaccccgctcttcgttcgcctag-5'              | 配列番号18 |
| 5'-gatcccgggcactgaagtatgaagtgaaggcgaag -3'                    | 配列番号19 |
| 3'- ggcccgtgacttcatacttcacgttccgcttccctag-5'                  | 配列番号20 |
| 5'-gatcctagtgaggagacattggaaatgtgaacatgtggactttgtgg-3'         | 配列番号21 |
| 3'- gatcaccctctgtaacctttacactgtacaccctgaaacaccctag-5'         | 配列番号22 |

## 【0012】

## 実施例3

融合酵素G308C1、G308C2、 G308C3、 G308C5及びG308C13の発現及び抽出

実施例2の組み換えDNA pUCMG308C1、 pUCMG308C3、 pUCMG308C5、 pUCMG308C2及びpUCMG308C13 を大腸菌XL1-Blueに形質転換した。Levyら (Protein Science, 1, 329-, 1992) の方法に従って、形質転換した大腸菌を37℃16時間LB培地 (DIFCO社) で振とう培養して得た培養液を、終濃度が2%となるようにLB培地に植菌し、37℃5時間振とう培養後、IPTG (Isopropyl-β-D-thiogalactopyranoside) を終濃度0.5mMで添加して発現を誘導し、37℃16時間培養した後、4℃、6000rpm

で10分間遠心分離することにより菌体を得た。これを 8% sucrose、0.1% Triton-X、50mM EDTAを含有する10mM Tris/HCl緩衝液に懸濁後、終濃度33mg/mlのリゾチームを添加して、37℃、30分間静置した後、15000rpm、20分間の遠心操作で、不溶物を取り除いて融合酵素G308C1、G308C2、G308C3、G308C5及びG308C13液をそれぞれ得た。なお、対照として、プラスミドpUCMGについても同様に行い、野生型酵素液を得た。

【 0 0 1 3 】

#### 実施例 4

融合酵素G308C1、G308C2、G308C3、G308C5及びG308C13の酵素活性に対する抗CRP抗体の効果

実施例3で得た融合酵素液の活性を、抗CRP抗体の非存在及び存在下でそれぞれ測定し、抗CRP抗体結合による酵素活性の変動を調べた。各融合酵素液を1%牛アルブミン、3mM塩化マグネシウム、150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8)で400倍希釈したもの6 $\mu$ lに、3mM塩化マグネシウムと150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8; 以下、緩衝液Aと省略する)、または抗CRPヤギ抗体を緩衝液Aで100倍希釈した抗体液を150 $\mu$ l添加し、37℃で5分間反応後、10mM グルコース-6-リン酸(G6P)、6mM ニコチンアミドアデニンジヌクレオチド(NAD)を含有する緩衝液A 75 $\mu$ lを添加し、37℃で5分間反応させ、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた。抗CRP抗体非存在時の酵素活性に対する、抗体存在時の活性を活性比として表2に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素G308C1、G308C2、G308C3、G308C5及びG308C13では、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが判る。すなわち、G6PDHの Pro308/Ala309間に配列番号2、3、4、5に記載のCRPペプチド 或いはその一部を挿入した融合酵素では、抗CRP抗体の結合により、その酵素活性が阻害されることが判る。

【表 2】

|        | 活性比  |
|--------|------|
| 野生型    | 101% |
| 融合酵素   |      |
| G308C1 | 86%  |
| G308C2 | 29%  |
| G308C3 | 36%  |
| G308C5 | 88%  |
| G308CB | 39%  |

$$\text{活性比} = \frac{\text{抗体存在時のG6PDH活性}}{\text{抗体非存在時のG6PDH活性}} \times 100$$

## 実施例 5

## 融合酵素G308C1を用いた抗CRP抗体の測定

抗CRP抗体量依存的な融合酵素G308C1活性の変動を調べた。抗CRPモノクローナル抗体を緩衝液Aで100倍、1000倍、10000倍、100000倍希釈した各溶液あるいは緩衝液A 50  $\mu$  lに、緩衝液Aで3300倍希釈した融合酵素G308C1液を100  $\mu$  l添加し、37℃で5分間反応後、10mM G6P、6mM NADを含有する緩衝液A 75  $\mu$  lを添加して、37℃で5分間反応させて、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた結果を図1に示す。その結果から判るように、抗CRP抗体量の減少に伴い酵素活性が増加する現象が見られた。すなわち、G6PDHの Pro308/Ala309間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、抗CRP抗体が測定できることが示された。

【 0 0 1 4 】

## 実施例 6

## 融合酵素G308C1を用いたCRPの測定

抗CRP抗体の結合により阻害される融合酵素活性の、CRP濃度による回復を測定した。各種濃度 (0、10、20、40 mg/dl) のCRP溶液6  $\mu$  lに、緩衝液Aで3300倍希釈した融合酵素G308C1液を100  $\mu$  l添加し、37℃で3分間反応後、緩衝液Aで10000倍希釈した抗CRPモノクローナル抗体液50  $\mu$  lを添加した。さらに37℃で3分間反

応させ、10mM G6P、6mM NADを含有する緩衝液A 75  $\mu$ lを添加して、37℃で5分間反応させて、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた。図2に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHの Pro308/Ala309間に配列番号2に記載のCRP<sup>ペプチド</sup>を挿入した融合酵素を用いて、CRPが測定できることが示された。

#### 実施例 7

##### 融合酵素G308C2、G308C3、G308C5を用いたCRPの測定

実施例6と同様の方法により、CRPの測定を試みた。なお、融合酵素G308C2、G308C3、G308C5液の希釈は各々5000倍、15000倍、2000倍、抗CRPヤギ抗体の希釈倍率は全て8倍で実施した。その結果、図3、4、5に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのPro308/Ala309間に配列番号3、4、5に記載のCRP<sup>ペプチド</sup>を挿入した融合酵素を用いて、CRPが測定できることが示された。

【 0 0 1 5 】

#### 実施例 8

##### G6PDHのAsp306/Val307間に、CRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と23、8と24に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAsp306/Val307間にのみ制限酵素BamH I 配列を有する組み換え体 pUCMG306Bを構築し、配列番号13に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG306C1を構築した。

#### 実施例 9

##### 融合酵素G306C1を用いたCRPの測定

実施例3と同様の操作により、融合酵素G306C1液を得た。次いで、実施例6と同様の方法により、CRPの測定を試みた。なお、融合酵素液の希釈は500倍、抗CRPモノクローナル抗体の希釈倍率は3200倍で実施した。その結果、図6に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのAsp306/Val307間に配列番号2に記載のCRP<sup>ペプチド</sup>を挿入した融合酵素を用いて、CRPが測定できることが示された。

【 0 0 1 6 】

実施例10

G6PDHのAla309/Asp310間にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と25、8と26に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAla309/Asp310間にのみ制限酵素BamH I 配列を有する組み換え体 pUCMG309Bを構築し、配列番号13に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG309C1を構築した。

実施例11

融合酵素G309C1を用いたCRPの測定

実施例3と同様の操作により、融合酵素G309C1液を得た。次いで、実施例6と同様の方法により、CRPの測定を試みた。なお、融合酵素液の希釈は2500倍、抗CRPモノクローナル抗体の希釈倍率は10000倍で実施した。その結果、図7に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのAla309/Asp310間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、CRPが測定できることが示された。

【 0 0 1 7 】

実施例12

G6PDHのC末端にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

Kunkel法に従い、プラスミドpBSMG、配列番号27に示したオリゴヌクレオチドプライマー及び、Mutan-K（宝酒造社）を使用して、G6PDH遺伝子のC-末端に制限酵素BamH I 配列を付加したプラスミドpBSMGCBを得た。次いで、実施例1、2と同様の操作により、C-末端にのみ制限酵素BamH I 配列を有する組み換え体 pUCMGCBを構築し、配列番号13に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMGCC1を構築した。

実施例13

融合酵素GCC1の酵素活性に対する抗CRP抗体の効果

実施例3と同様の操作により、融合酵素GCC1液を得た。次いで、実施例4と同様

の操作により、抗CRP抗体が融合酵素GCC1に結合した場合の効果を調べた。抗CRP抗体非存在時の酵素活性に対する、抗体存在時の活性を活性比として表3に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素GCC1は、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが示された。すなわち、G6PDHのC末端に配列番号2に記載のCRPペプチドを連結した融合酵素では、抗CRP抗体の結合により、その酵素活性が阻害されることが示された。

【表 3】

|              | 活性比  |
|--------------|------|
| 野生型          | 101% |
| 融合酵素<br>GCC1 | 95%  |

$$\text{活性比}(\%) = \frac{\text{抗体存在時のG6PDH活性}}{\text{抗体非存在時のG6PDH活性}} \times 100$$

## 【 0 0 1 8 】

## 実施例14

G6PDHのGlu362/Gln363間にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と28、8と29に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のGlu362/Gln363間にのみ制限酵素BamH I 配列を有する組み換え体 pUCMG362Bを構築し、配列番号13に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG362C1を構築した。

## 実施例15

融合酵素G362C1を用いた抗CRP抗体の測定

実施例3と同様の操作により、融合酵素G362C1液を調製し、次いで、実施例5と同様の方法により抗CRP抗体の量による融合酵素G362C1活性の変動を調べた。その結果、図8に示したように、抗CRP抗体量の増加に伴い酵素活性が増加する

現象が見られた。すなわち、G6PDHのGlu362/Gln363間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、抗CRP抗体が測定できることが示された。

【 0 0 1 9 】

【発明の効果】

本発明の外来ペプチドが挿入されたハイブリッド酵素を用いれば、当該外来ペプチドに結合能を有する物質の存在又はその量を直接に測定することができ、また、当該ハイブリッド酵素と当該外来ペプチドに結合能を有する物質とを組み合わせ用いれば、当該外来ペプチドを含む高分子物質の存在または量を間接的に検出・測定することが出来、さらに本発明のハイブリッド酵素を用いることで、ホモジニアスな比色法で試料中の微量なCRP測定が可能となり、組織中の炎症の早期発見、ひいては早期の病気の診断が可能となり、また各種高分子物質をホモジニアスな系で容易に測定することができる。

【 0 0 2 0 】

【配列表】

SEQUENCE LISTING

<110> WAKO PURE CHEMICAL INDUSTRIES, LTD.

<120> Hybrid Enzymes and Use Thereof

<130> WJ017

<140>

<141>

<160> 31

<170> PatentIn Ver. 2.1

<210> 1

<211> 206

<212> PRT

<213> Human

<400> 1

Gln Thr Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp

1

5

10

15

Thr Ser Tyr Val Ser Leu Lys Ala Pro Leu Thr Lys Pro Leu Lys Ala

20

25

30

Phe Thr Val Cys Leu His Phe Tyr Thr Glu Leu Ser Ser Thr Arg Gly

35

40

45

Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg Gln Asp Asn Glu Ile Leu

50

55

60

Ile Phe Trp Ser Lys Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser

65

70

75

80

Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val Ala Pro Val His Ile

85

90

95

Cys Thr Ser Trp Glu Ser Ala Ser Gly Ile Val Glu Phe Trp Val Asp

100

105

110

Gly Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly Tyr Thr Val Gly



|   |     |     |     |
|---|-----|-----|-----|
| 115   | 120 | 125 |     |
| Ala Glu Ala Ser Ile Ile Leu Gly Gln Glu Gln Asp Ser Phe Gly Gly |     |     |     |
| 130   | 135 | 140 |     |
| Asn Phe Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Gly Asn Val Asn |     |     |     |
| 145   | 150 | 155 | 160 |
| Met Trp Asp Phe Val Leu Ser Pro Asp Glu Ile Asn Thr Ile Tyr Leu |     |     |     |
| 165   |     | 170 | 175 |
| Gly Gly Pro Phe Ser Pro Asn Val Leu Asn Trp Arg Ala Leu Lys Tyr |     |     |     |
| 180   |     | 185 | 190 |
| Glu Val Gln Gly Glu Val Phe Thr Lys Pro Gln Leu Trp Pro         |     |     |     |
| 195   |     | 200 | 205 |

<210> 2

<211> 16

<212> PRT

<213> Human

<400> 2

|   |   |    |    |
|---|---|----|----|
| Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp Thr Ser |   |    |    |
| 1   | 5 | 10 | 15 |

<210> 3

<211> 27

<212> PRT

<213> Human

<400> 3

Leu Val Gly Asp Ile Gly Asn Val Asn Met Trp Asp Phe Val Leu Ser

1

5

10

15

Pro Asp Glu Ile Asn Thr Ile Tyr Leu Gly Gly

20

25

<210> 4

<211> 12

<212> PRT

<213> Human

<400> 4

Leu Lys Lys Gly Tyr Thr Val Gly Ala Glu Ala Ser

1

5

10

<210> 5

<211> 10

<212> PRT

<213> Human

<400> 5

Arg Ala Leu Lys Tyr Glu Val Gln Gly Glu

1                      5                      10

<210> 6

<211> 486

<212> PRT

<213> *Leuconostoc mesenteroides*

<400> 6

Met Val Ser Glu Ile Lys Thr Leu Val Thr Phe Phe Gly Gly Thr Gly

1                      5                      10                      15

Asp Leu Ala Lys Arg Lys Leu Tyr Pro Ser Val Phe Asn Leu Tyr Lys

20                      25                      30

Lys Gly Tyr Leu Gln Lys His Phe Ala Ile Val Gly Thr Ala Arg Gln

35                      40                      45

Ala Leu Asn Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys

50                      55                      60

Asp Phe Thr Asp Asp Gln Ala Gln Ala Glu Ala Phe Ile Glu His Phe

65                      70                      75                      80

Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Ala Val Leu

85                      90                      95

Lys Glu Ala Ile Glu Glu Ala Ala Asp Lys Phe Asp Ile Asp Gly Asn

100                      105                      110

Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala

115

120

125

Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Asp Thr Gly Tyr Asn Arg

130

135

140

Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Asp Thr Ala Ala Glu

145

150

155

160

Leu Gln Asn Asp Leu Glu Asn Ala Phe Asp Asp Asn Gln Leu Phe Arg

165

170

175

Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu

180

185

190

Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile

195

200

205

Lys Asn Val Gln Val Thr Leu Ser Glu Val Leu Gly Val Glu Glu Arg

210

215

220

Ala Gly Tyr Tyr Asp Thr Ala Gly Ala Leu Leu Asp Met Ile Gln Asn

225

230

235

240

His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser

245

250

255

Phe Thr Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala

260

265

270

Leu Lys Ile Tyr Asp Glu Ala Glu Val Asn Lys Tyr Phe Val Arg Ala

275

280

285

Gln Tyr Gly Ala Gly Asp Ser Ala Asp Phe Lys Pro Tyr Leu Glu Glu

290

295

300

Leu Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu

305

310

315

320

Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg

325

330

335

Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe

340

345

350

Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ala

355

360

365

Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu

370

375

380

Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asp Leu

385

390

395

400

Gly Trp Thr Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr

405

410

415

Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala  
420 425 430

Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Ser  
435 440 445

Ala Val Tyr Thr Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly  
450 455 460

Ser Met Gly Pro Glu Ala Ser Asp Lys Leu Leu Ala Ala Asn Gly Asp  
465 470 475 480

Ala Trp Val Phe Lys Gly  
485

<210> 7

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 7

ataaggggta caccatgggtt tcagaaatca agacgtag

39

<210> 8

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 8

ttcccgggct ttaattaacc tttaaacacc 30

<210> 9

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 9

tggttggtta gctatggaaa aaccagaatc 30

<210> 10

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 10

taggatccag gtacgtctaa ttcttcaagg tatg

34

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 11

atggatccgc tgattctaaa aacaatacct tc

32

<210> 12

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 12

aagcttgcat gcctgcaggt tcccg

25



<210> 13

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 2, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 13

gatccgacat gtcgaggaag gcttttgtgt ttcccaaaga gtcggatact tccg 54

<210> 14

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 1.  
3

<400> 14

gatccggaag tatccgactc ttgaggaaac acaaaagcct tcctcgacat gtcg 54

<210> 15

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 15

gatccgtgct gtcaccagat gagattaaca ccattctatct tggcgggg 48

<210> 16

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 1  
5

<400> 16

gatccccgc caagatagat ggtgttaatc tcattctggtg acagcacg 48

<210> 17

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 4, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 17

gatccctgaa gaagggtac actgtggggg cagaagcaag cg

42

<210> 18

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 1  
7

<400> 18

gatccgcttg cttctgcccc cacagtgtat cccttcttca gg

42

<210> 19

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 5, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 19

gatcccgggc actgaagtat gaagtgcaag gcgaag

36

<210> 20

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 1  
9

<400> 20

gaccccttcgc cttgcacttc atacttcagt gcccg

36

<210> 21

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of

the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 21

gatcctagtg ggagacattg gaaatgtgaa catgtgggac tttgtgg

47

<210> 22

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 2  
1

<400> 22

gatccacaaa agtccacat gttcacattt ccaatgtctc ccactag

47

<210> 23

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 23

taggatccgt ctaattcttc aaggtatggc ttg

33

<210> 24

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 24

aaggatccgt acctgctgat tctaaaaaca atac

34

<210> 25

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<400> 25

ttggatccag caggtacgtc taattcttca ag

32

<210> 26

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 26

taggatccga ttctaaaaac aataccttca tcg 33

<210> 27

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 27

gggtgtttaa aggtggatcc taattaaagc ccgg 34

<210> 28

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 28

taggatacctt ctgaaccaaa gttaaactg cc

32

<210> 29

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 29

atggatccca agaagcacia gaagctgtct tg

32

<210> 30

<211> 1024

<212> PRT

<213> Escherichia coli

<400> 30

Met Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Arg Asp

1

5

10

15

Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro

20

25

30

Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro



35

40

45

Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg Phe Ala Trp Phe

50

55

60

Pro Ala Pro Glu Ala Val Pro Glu Ser Trp Leu Glu Cys Asp Leu Pro

65

70

75

80

Glu Ala Asp Thr Val Val Val Pro Ser Asn Trp Gln Met His Gly Tyr

85

90

95

Asp Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro Ile Thr Val Asn Pro

100

105

110

Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys Tyr Ser Leu Thr Phe

115

120

125

Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr Arg Ile Ile Phe

130

135

140

Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys Asn Gly Arg Trp Val

145

150

155

160

Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu Phe Asp Leu Ser Ala

165

170

175

Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val Met Val Leu Arg Trp

180

185

190

Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met Trp Arg Met Ser Gly  
195 200 205

Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro Thr Thr Gln Ile Ser  
210 215 220

Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp Phe Ser Arg Ala Val  
225 230 235 240

Leu Glu Ala Glu Val Gln Met Cys Gly Glu Leu Arg Asp Tyr Leu Arg  
245 250 255

Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln Val Ala Ser Gly Thr  
260 265 270

Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg Gly Gly Tyr Ala Asp  
275 280 285

Arg Val Thr Leu Arg Leu Asn Val Glu Asn Pro Lys Leu Trp Ser Ala  
290 295 300

Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu Leu His Thr Ala Asp  
305 310 315 320

Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val Gly Phe Arg Glu Val  
325 330 335

Arg Ile Glu Asn Gly Leu Leu Leu Leu Asn Gly Lys Pro Leu Leu Ile  
340 345 350

Arg Gly Val Asn Arg His Glu His His Pro Leu His Gly Gln Val Met

355

360

365

Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu Met Lys Gln Asn Asn

370

375

380

Phe Asn Ala Val Arg Cys Ser His Tyr Pro Asn His Pro Leu Trp Tyr

385

390

395

400

Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val Asp Glu Ala Asn Ile

405

410

415

Glu Thr His Gly Met Val Pro Met Asn Arg Leu Thr Asp Asp Pro Arg

420

425

430

Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg Met Val Gln Arg Asp

435

440

445

Arg Asn His Pro Ser Val Ile Ile Trp Ser Leu Gly Asn Glu Ser Gly

450

455

460

His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp Ile Lys Ser Val Asp

465

470

475

480

Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly Ala Asp Thr Thr Ala

485

490

495

Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val Asp Glu Asp Gln Pro

500

505

510

Phe Pro Ala Val Pro Lys Trp Ser Ile Lys Lys Trp Leu Ser Leu Pro

515

520

525

Gly Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr Ala His Ala Met Gly

530

535

540

Asn Ser Leu Gly Gly Phe Ala Lys Tyr Trp Gln Ala Phe Arg Gln Tyr

545

550

555

560

Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp Val Asp Gln Ser Leu

565

570

575

Ile Lys Tyr Asp Glu Asn Gly Asn Pro Trp Ser Ala Tyr Gly Gly Asp

580

585

590

Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys Met Asn Gly Leu Val

595

600

605

Phe Ala Asp Arg Thr Pro His Pro Ala Leu Thr Glu Ala Lys His Gln

610

615

620

Gln Gln Phe Phe Gln Phe Arg Leu Ser Gly Gln Thr Ile Glu Val Thr

625

630

635

640

Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu Leu Leu His Trp Met

645

650

655

Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly Glu Val Pro Leu Asp

660

665

670

Val Ala Pro Gln Gly Lys Gln Leu Ile Glu Leu Pro Glu Leu Pro Gln

675

680

685

Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val Arg Val Val Gln Pro

690

695

700

Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile Ser Ala Trp Gln Gln

705

710

715

720

Trp Arg Leu Ala Glu Asn Leu Ser Val Thr Leu Pro Ala Ala Ser His

725

730

735

Ala Ile Pro His Leu Thr Thr Ser Glu Met Asp Phe Cys Ile Glu Leu

740

745

750

Gly Asn Lys Arg Trp Gln Phe Asn Arg Gln Ser Gly Phe Leu Ser Gln

755

760

765

Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr Pro Leu Arg Asp Gln

770

775

780

Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Ala Thr

785

790

795

800

Arg Ile Asp Pro Asn Ala Trp Val Glu Arg Trp Lys Ala Ala Gly His

805

810

815

Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr Ala Asp Thr Leu Ala

820

825

830

Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp Gln His Gln Gly Lys

835

840

845

Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile Asp Gly Ser Gly Gln

850

855

860

Met Ala Ile Thr Val Asp Val Glu Val Ala Ser Asp Thr Pro His Pro

865

870

875

880

Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln Val Ala Glu Arg Val

885

890

895

Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr Pro Asp Arg Leu Thr

900

905

910

Ala Ala Cys Phe Asp Arg Trp Asp Leu Pro Leu Ser Asp Met Tyr Thr

915

920

925

Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg Cys Gly Thr Arg Glu

930

935

940

Leu Asn Tyr Gly Pro His Gln Trp Arg Gly Asp Phe Gln Phe Asn Ile

945

950

955

960

Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr Ser His Arg His Leu

965

970

975

Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile Asp Gly Phe His Met

980

985

990

Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Phe

995

1000

1005

Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys

1010

1015

1020

<210> 31

<211> 448

<212> PRT

<213> Escherichia coli

<400> 31

Thr Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile

1

5

10

15

Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala Ala

20

25

30

Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu

35

40

45

Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr

50

55

60

Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu

65

70

75

80

Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro

85

90

95

Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly

100

105

110

Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp

115

120

125

His Pro Thr Ile Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr Gly

130

135

140

Asn Val Ser Thr Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala Leu Val

145

150

155

160

Ala His Val Thr Ser Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu

165

170

175

Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr

180

185

190

Glu Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala



195

200

205

Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr

210

215

220

Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala

225

230

235

240

Ala Ser Leu Asn Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu

245

250

255

Gly Leu Phe Ala Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro Lys

260

265

270

Ala Thr Tyr His Gly Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro

275

280

285

Asn Pro Gln Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp

290

295

300

Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln

305

310

315

320

Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys

325

330

335

Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala

340

345

350

Leu Glu Phe Ala Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala  
355 360 365

Asp His Ala His Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala Pro  
370 375 380

Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp Gly Ala Val Met Val Met  
385 390 395 400

Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Gln Leu  
405 410 415

Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr  
420 425 430

Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys  
435 440 445

# 【 0 0 2 1 】

## 【図面の簡単な説明】

【図 1】 本発明のハイブリッド酵素 G 3 0 8 C 1 の抗 C R P 抗体測定能を示すグラフである。。

【図 2】 本発明のハイブリッド酵素 G 3 0 8 C 1 の C R P 測定能を示すグラフである。なお、図 2 から 8 において

回復率(%) = {(各 CRP 濃度時の G6PDH 活性) - (CRP 0mg/dl 時の G6PDH 活性)} / {(抗体非存在時の G6PDH 活性) - (CRP 0mg/dl 時の G6PDH 活性)} X100

活性比(%) = {抗体存在時の G6PDH 活性 / 抗体非存在時の G6PDH 活性} X100 である

。

【図 3】 本発明のハイブリッド酵素 G 3 0 8 C 2 の C R P 測定能を示すグラフである。

【図 4】 本発明のハイブリッド酵素 G 3 0 8 C 3 の C R P 測定能を示すグラフである。

【図 5】 本発明のハイブリッド酵素 G 3 0 8 C 5 の C R P 測定能を示すグラフである。

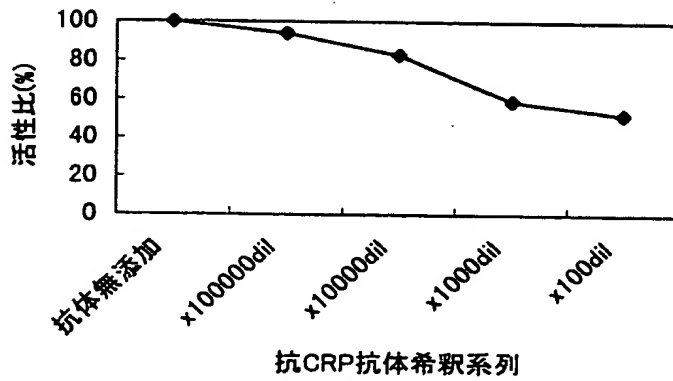
【図 6】 本発明のハイブリッド酵素 G 3 0 6 C 1 の C R P 測定能を示すグラフである。

【図 7】 本発明のハイブリッド酵素 G 3 0 9 C 1 の C R P 測定能を示すグラフである。

【図 8】 本発明のハイブリッド酵素 G 3 6 2 C 1 の抗 C R P 抗体測定能を示すグラフである。

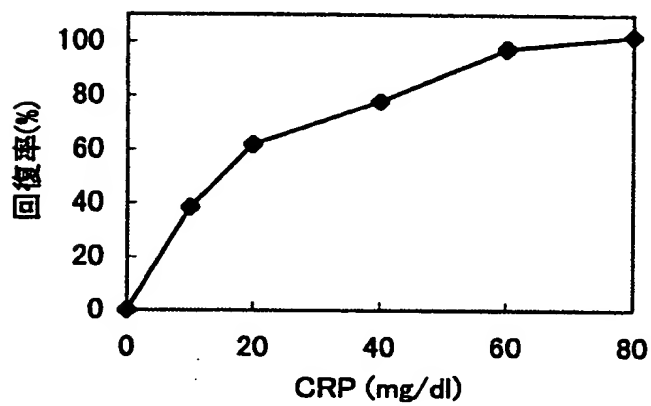
【書類名】 図面

【図 1】

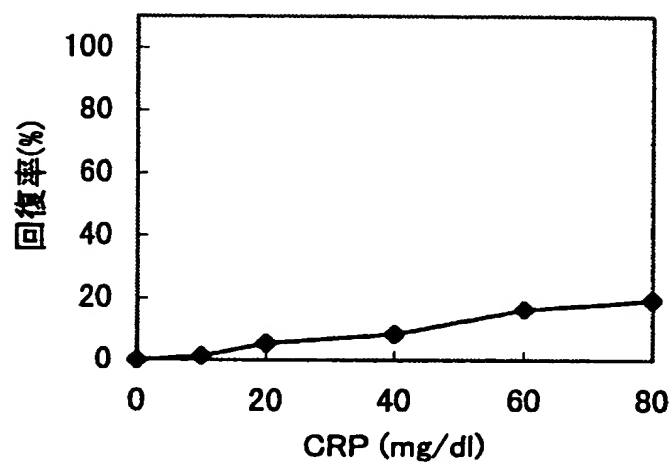


$$\text{活性比} = \frac{\text{抗体存在時のG6PDH活性}}{\text{抗体非存在時のG6PDH活性}} \times 100 \text{ (\%)}$$

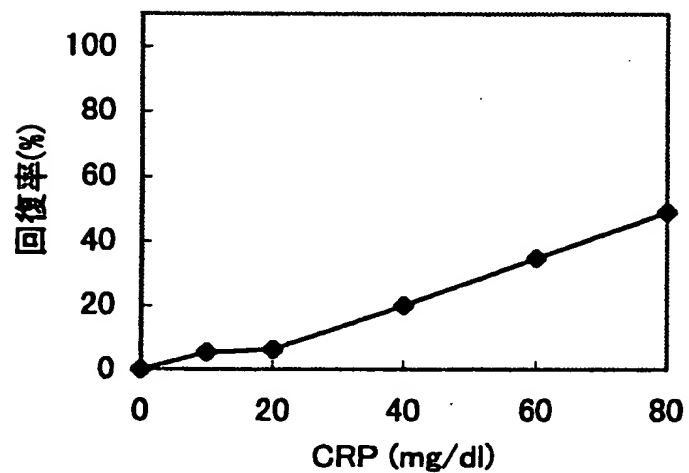
【図 2】



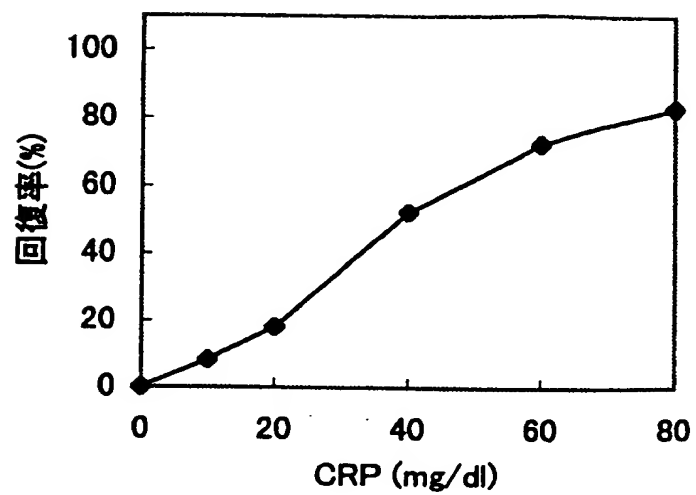
【図 3】



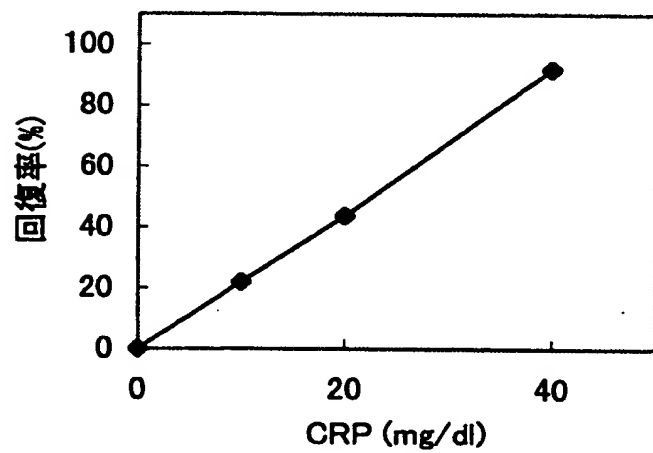
【図 4】



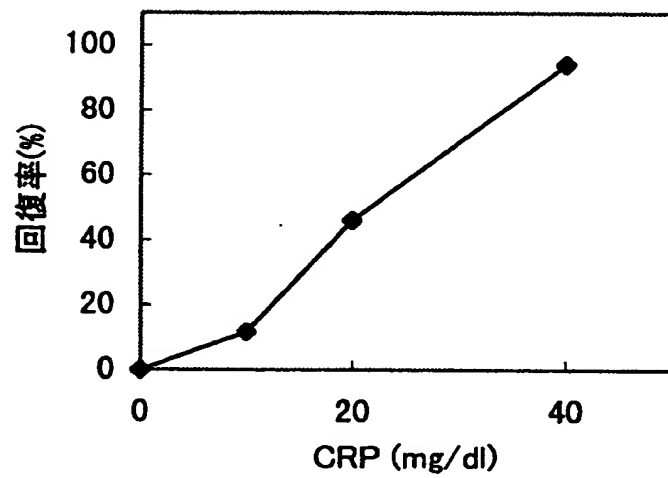
【図 5】



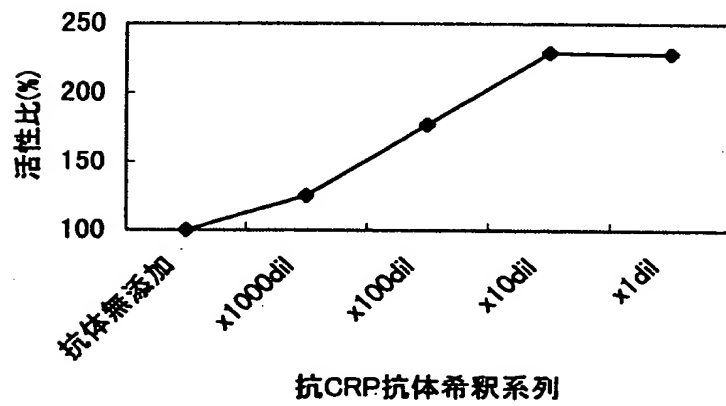
【図 6】



【図 7】



【図 8】



【書類名】 要約書

【要約】

【課題】 ホモジニアスな比色法で試料中の微量な C R P 測定を可能とする方法を提供すること、および高分子物質をホモジニアスな系で測定する方法を提供することである。

【解決手段】 G6PDH に於ける、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、特定位置を見出し、当該位置に C R P 由来のペプチドを挿入しハイブリッド酵素を提供し、このものと試料とを接触させたときの酵素活性の変化をみることにより、ホモジニアスな比色法で試料中の微量な C R P 測定を可能とした。

【選択図】 なし



認定・付加情報

|         |                  |
|---------|------------------|
| 特許出願の番号 | 特願 2000-174604   |
| 受付番号    | 50000722895      |
| 書類名     | 特許願              |
| 担当官     | 仲村 百合子 1730      |
| 作成日     | 平成 12 年 6 月 16 日 |

<認定情報・付加情報>

|          |                                  |
|----------|----------------------------------|
| 【提出日】    | 平成12年 6月12日                      |
| 【特許出願人】  |                                  |
| 【識別番号】   | 000252300                        |
| 【住所又は居所】 | 大阪府大阪市中央区道修町3丁目1番2号              |
| 【氏名又は名称】 | 和光純薬工業株式会社                       |
| 【代理人】    | 申請人                              |
| 【識別番号】   | 100089314                        |
| 【住所又は居所】 | 東京都港区西新橋2丁目3番2号 ニュー栄和ビル 大多和特許事務所 |
| 【氏名又は名称】 | 大多和 明敏                           |
| 【代理人】    |                                  |
| 【識別番号】   | 100086999                        |
| 【住所又は居所】 | 東京都港区西新橋2丁目3番2号 ニュー栄和ビル 大多和特許事務所 |
| 【氏名又は名称】 | 大多和 曉子                           |

出 願 人 履 歴 情 報

識別番号 [000252300]

1. 変更年月日 1990年 8月 7日

[変更理由] 新規登録

住 所 大阪府大阪市中央区道修町3丁目1番2号  
氏 名 和光純薬工業株式会社

J A P A N   P A T E N T   O F F I C E

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application       : June 12, 2000  
Application Number       : No.174604/2000  
Applicant                 : Wako Pure Chemical Industries, Ltd.

May 11, 2001

Commissioner,  
Japan Patent Office

Kozo Oikawa

Certificate No. 2001-3038775

|  |  |
|--|--|
| <b>【NAME OF DOCUMENT】</b>                    | Patent request   |
| <b>【REFERENCE NUMBER】</b>                    | WJ017  |
| <b>【ADDRESSEE】</b>                           | To the Commissioner of the JPO   |
| <b>【INTERNATIONAL PATENT CLASSIFICATION】</b> |  |
|  | C12N 9/00  |
|  | C12N 15/52   |
|  | G01N 33/50   |
| <b>【INVENTOR DOMICILE NAME】</b>              |  |
| <b>【DOMICILE】</b>                            | 6-1, Takada-cho, Amagasaki-shi, Hyogo<br>Wako Pure Chemical Industries, LTD in<br>Osaka Laboratory |
| <b>【NAME】</b>                                | Sachiko Yamamoto   |
| <b>【INVENTOR DOMICILE NAME】</b>              |  |
| <b>【DOMICILE】</b>                            | 6-1, Takada-cho, Amagasaki-shi, Hyogo<br>Wako Pure Chemical Industries, LTD in<br>Osaka Laboratory |
| <b>【NAME】</b>                                | Minoru Shiro   |
| <b>【INVENTOR DOMICILE NAME】</b>              |  |
| <b>【DOMICILE】</b>                            | 6-1, Takada-cho, Amagasaki-shi, Hyogo<br>Wako Pure Chemical Industries, LTD in<br>Osaka Laboratory |
| <b>【NAME】</b>                                | Toshiro Hanada   |
| <b>【INVENTOR DOMICILE NAME】</b>              |  |
| <b>【DOMICILE】</b>                            | 6-1, Takada-cho, Amagasaki-shi, Hyogo<br>Wako Pure Chemical Industries, LTD in<br>Osaka Laboratory |
| <b>【NAME】</b>                                | Shinzo Kobatake  |

**[APPLICANT FOR PATENT DOMICILE NAME]**

**[IDENTIFICATION NUMBER]** 000252300

**[NAME]** Wako Pure Chemical Industries, Ltd.

**[AGENT]**

**[IDENTIFICATION NUMBER]** 100089314

**[PATENT ATTORNEY]**

**[NAME]** Akitoshi Ottawa

**[AGENT]**

**[IDENTIFICATION NUMBER]** 100086999

**[PATENT ATTORNEY]**

**[NAME]** Akiko Ottawa

**[INDICATION OF FEE]**

**[DEPOSIT ACCOUNT NUMBER]** 014742

**[FEE]** 21,000yen

**[LIST OF ANNEXED DOCUMENT]**

**[DOCUMENT]** Specification 1

**[DOCUMENT]** Drawing 1

**[DOCUMENT]** Abstract 1

**[REQUIREMENT OF PROOF]** Request

【Title of Document】 specification

【Title of the Invention】 HYBRID ENZYME AND USE THEREOF

【Scope of Claim for a Patent】

【Claim 1】 A hybrid enzyme which has a partial substitution or an insertion of a peptide containing a part of an amino acid sequence represented by SEQ ID NO:1, in which said hybrid enzyme has the same enzyme activity as an original enzyme without the substitution or the insertion of said peptide, and said hybrid enzyme activity is modulated when a material having binding ability to said peptide introduced by the substitution or the insertion is bound to the peptide moiety.

【Claim 2】 The hybrid enzyme according to claim 1, in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from the amino acid sequence of SEQ ID NO: 1.

【Claim 3】 The hybrid enzyme according to claim 2, in which the peptide has a property of being capable of binding to a material having binding ability to C-reactive protein.

【Claim 4】 The hybrid enzyme according to claim 1, in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from any one of SEQ ID NO: 2 through SEQ ID NO: 5.

【Claim 5】 The hybrid enzyme according to claim 1, in which the original enzyme is a glucose-6-phosphate dehydrogenase, a  $\beta$ -galactosidase or an alkaline phosphatase.

【Claim 6】 The hybrid enzyme according to claim 1, in which the material having binding ability to the peptide is an antibody.

【Claim 7】 A reagent for measurement of C-reactive protein comprising the hybrid enzyme according to any one of claims 1 through 6.

【Claim 8】 The reagent according to claim 7 further comprising an anti-C-reactive protein antibody.

【Claim 9】 A kit for measurement of C-reactive protein containing a reagent

comprising the hybrid enzyme according to any one of claims 1 through 6.

【Claim 10】 The kit according to claim 9 further comprising an anti-C-reactive protein antibody.

【Claim 11】 A method for measurement of C-reactive protein which is characterized in using the hybrid enzyme according to any one of claims 1 through 6.

【Claim 12】 The method according to claim 11 further comprising using an anti-C-reactive protein antibody in combination.

【Claim 13】 A method for measurement of C-reactive protein comprising bringing a sample containing C-reactive protein, the enzyme according to any one of claims 1 through 6 and an anti-C-reactive protein antibody into contact with one another, then measuring activity of the enzyme, and determining the amount of C-reactive protein in the sample based on the resulting enzyme activity.

【Claim 14】 A glucose-6-phosphate dehydrogenase, into which a peptide is introduced at a specific position by insertion or substitution.

【Claim 15】 The hybrid enzyme according to claim 14, in which the specific position is a position at which the glucose-6-phosphate dehydrogenase activity can be maintained even by the insertion or substitution of a peptide having 6 or more amino acid residues.

【Claim 16】 The hybrid enzyme according to claim 14, in which the specific position is a position at which the glucose-6-phosphate dehydrogenase activity is modulated when a material having binding ability to the peptide introduced by insertion or substitution is bound to said peptide.

【Claim 17】 The hybrid enzyme according to claim 14, in which the specific position is any position selected from the group consisting of the Leu305 to Asp310 positions, the Glu362 position and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

【Claim 18】 The hybrid enzyme according to claim 14, in which the peptide is

selected from the amino acid sequence of C-reactive protein.

【Claim 19】 The hybrid enzyme according to claim 14, in which the peptide has a character that there is a material having binding ability specifically to the peptide.

【Claim 20】 A reagent for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 21】 A kit for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 22】 A method for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which is characterized in using the hybrid enzyme according to any one of claims 14 through 19.

【Claim 23】 A method for measurement of a material containing the peptide, which comprises using the hybrid enzyme according to any one of claims 14 through 19 in combination with a material having binding ability to the peptide introduced into the hybrid enzyme by insertion or substitution.

【Claim 24】 A method for measurement of a material containing said peptide introduced into the hybrid enzyme according to any one of claim 14 through 19, which comprises bringing the hybrid enzyme according to any one of claims 14 through 19, a sample containing a material containing the peptide introduced into said hybrid enzyme by insertion or substitution and a material having binding ability to said peptide into contact with one another, then measuring activity of said hybrid enzyme, and determining the amount of the material containing said peptide in the sample based on the resulting enzyme activity.



**【Claim 25】** A reagent for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

**【Claim 26】** A kit for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

**【Claim 27】** A method for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises using the hybrid enzyme according to any one of claims 14 through 19.

**【Claim 28】** A method for measurement of a material having binding ability to said peptide introduced into the hybrid enzyme according to any one of claim 14 through 19, which comprises bringing the hybrid enzyme according to any one of claims 14 through 19 into contact with a sample containing a material having binding ability to the peptide, then measuring an activity of said hybrid enzyme, and determining the amount of the material having binding ability to said peptide in the sample based on the resulting enzyme activity.

**【Claim 29】** A gene coding for a hybrid enzyme comprising an amino acid sequence into which a foreign peptide is introduced by substitution or insertion at any position selected from the group consisting of the Leu305 to Asp310 positions, the Glu362 position and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

**【Claim 30】** A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 29 into a vector DNA.

**【Claim 31】** A transformant or a transductant comprising the recombinant DNA

according to claim 30.

**【Claim 32】** A method for producing a protein having enzyme activity of glucose-6-phosphate dehydrogenase and a property that the glucose-6-phosphate dehydrogenase activity is modulated when a material having binding ability to an amino acid sequence introduced into glucose-6-phosphate dehydrogenase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 31, and collecting the protein.

**【Claim 33】** A gene coding for a hybrid enzyme comprising an amino acid sequence into which an amino acid sequence, which can be cleaved with a restriction enzyme, is introduced by substitution or insertion at any position selected from the group consisting of the Leu305 to Asp310 positions, the Glu362 position and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

**【Claim 34】** A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 33 into a vector DNA.

**【Claim 35】** A  $\beta$ -galactosidase into which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced at a specific position by insertion or substitution.

**【Claim 36】** The hybrid enzyme according to claim 35, in which the specific position is either selected from the Ile280 to Asp281 positions and Val796 to Ser797 positions of an amino acid sequence of a  $\beta$ -galactosidase represented by SEQ ID NO: 30.

**【Claim 37】** A gene coding for the hybrid enzyme according to claim 36.

**【Claim 38】** A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 37 into a vector DNA.

**【Claim 39】** A transformant or a transductant comprising the recombinant DNA according to claim 38.

**【Claim 40】** A method for producing a protein having an enzyme activity of a  $\beta$ -

galactosidase and a property that the  $\beta$ -galactosidase activity is modulated when a material having binding ability to an amino acid sequence introduced into the  $\beta$ -galactosidase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 39, and collecting the protein.

[Claim 41] An alkaline phosphatase into which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced at a specific position by insertion or substitution.

[Claim 42] The hybrid enzyme according to claim 41, in which the specific position is any one selected from the Lys167 to Cys168 positions, the Cys168 to Tyr169 positions, the Glu407 to Asp408 positions, the Lys91 to Thr93 positions and the Tyr169 to Lys177 positions of an amino acid sequence of an alkaline phosphatase represented by SEQ ID NO: 31.

[Claim 43] A gene coding for the hybrid enzyme according to claim 42.

[Claim 44] A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 43 into a vector DNA.

[Claim 45] A transformant or a transductant comprising the recombinant DNA according to claim 44.

[Claim 46] A method for producing a protein having an enzyme activity of an alkaline phosphatase and a property that the alkaline phosphatase activity is modulated when a material having binding ability to an amino acid sequence introduced into the alkaline phosphatase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 45, and collecting the protein.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to a hybrid enzyme and a biological substance assay system using the same. More particularly, the present invention relates to a hybrid enzyme containing a part of an amino acid sequence of C-reactive protein (hereinafter briefly referred to as CRP) and CRP assay system using the hybrid enzyme, and a hybrid type of glucose-6-phosphate dehydrogenase (G6PDH) containing an foreign peptide at a specific position and a various foreign peptide assay system using the hybrid type enzyme.

[0002]

**[Prior Art]**

Biological substance assay systems utilizing the antigen-antibody reaction can be roughly divided into two types: homogeneous assay systems such as turbidimetric immunoassay and latex turbidimetric immunoassay, and heterogeneous assay systems such as an immunoassay using HPLC, electrophoresis etc., radio immunoassay and enzyme immunoassay (EIA). In either case, measurements are made at present using almost automated instruments. However, in the heterogeneous assay systems such as a macromolecule material assays or a small molecule material assays, special instruments are indispensable because solid phases are used. Accordingly, general-purpose automatic analyzers can not be applied to these systems. Also in the homogeneous assay systems, except for turbidimetric immunoassay and latex turbidimetric immunoassay, special instruments are used, and general-purpose automatic analyzers can not be applied to these systems. On the other hand, the EMIT method (IMMUNOASSAYS, R. M. Nakamura et al., Alan R. Liss, Inc., New York) using an enzyme is established and comes in practice at present as a homogeneous immunoassay system applicable to the general-purpose automatic analyzers. This assay system is a method for measurement of the amount of a material to be assayed by using an enzyme chemically modified with the material to be assayed and utilizing that enzyme activity is inhibited to result in a reduction in enzyme activity when an antibody to the material to

be assayed is bound thereto. However, this system has the problem that the material to be assayed is limited to a small molecule material such as haptene. Then, recently, a homogeneous assay system using a hybrid enzyme formed by inserting a foreign peptide into an enzyme by genic recombination is contrived as a homogeneous immunoassay system which makes it possible to assay a macromolecule material by use of the enzyme. That is to say, this is a method for assaying the amount of the foreign peptide, a material containing the peptide in its amino acid sequence, or a material having binding ability to the peptide, by utilizing that enzyme activity varies when an antibody is bound to a foreign peptide moiety in the hybrid enzyme. As the enzymes used in this method, there are known alkaline phosphatases (Published Japanese Translation of PCT International Publication (Toku-Hyo-Hei) 8-507686 (1996)) and  $\beta$ -galactosidases (FEBS Letters, 434, 23-27 (1998), FEBS Letters, 438, 267-271 (1998)). In this method, however, it is also known that the hybrid enzyme sometimes loses enzyme activity according to the position where a foreign peptide is inserted, or that even when the hybrid enzyme has enzyme activity, the enzyme activity sometimes does not vary when the material having binding ability is bound to the hybrid enzyme.

Meanwhile, CRP, which is  $\beta$ -globulin in the serum which reacts with C polysaccharide of *Streptococcus Pneumoniae*, is known to be acute phase reactant protein and to increase in an inflammation or tissue injury. The measurement of CRP is useful for early diagnosis of inflammation in tissue and diagnosis of diseases. Conventionally, CRP has been measured by turbidimetric immunoassay, latex turbidimetric immunoassay, enzyme immunoassay or radio immunoassay. Of these, turbidimetric immunoassay and latex turbidimetric immunoassay are applicable to the general-purpose automatic analyzers. However, as both are a method for detecting changes in turbidity, they are not said to be a complete homogeneous assay systems and have a problem with regard to accuracy on measurement. Accordingly, it has been desired to develop the micromasurement of CRP applicable to the general-purpose

automatic analyzers according to the complete homogeneous assay system with high in assay accuracy.

[0003]

[Problems to be Solved by the Invention]

In view of the situation as described above, one of problems to be solved by the present invention is to provide a method which makes it possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry. It is another problem to be solved by the present invention to provide a method for measuring a macromolecule material in a homogeneous system.

[0004]

[Means for Solving the Problems]

As a result of intensive research for solving the above-mentioned problems, the present inventors have found that the use of the above-mentioned hybrid enzyme makes it possible to assay in a homogeneous system a macromolecule material to be assayed, and have considered to be capable of developing a new CRP micromeasuring method applicable to the general-purpose automatic analyzers by application of this method to the assay of CRP, because the detection thereof can be conducted by a colorimetry. The present inventors have conducted further research, and have discovered that the above-mentioned problems can be solved by using a hybrid enzyme containing a part of a material to be analyzed such as CRP at a certain position of some kind of enzyme, thus completing the present invention.

That is to say, as an effective hybrid enzyme for providing a method which makes it possible to measure a trace amount of CRP in a sample by a homogeneous colorimetry using a hybrid enzyme in which a CRP-derived peptide is inserted into a specific position, the present invention provides (1) a hybrid enzyme which has a partial substitution or an insertion of a peptide containing a part of an amino acid sequence represented by SEQ ID NO:1, in which said hybrid enzyme has the same enzyme

activity as an original enzyme without the substitution or the insertion of said peptide, and said hybrid enzyme activity varied when a material having binding ability to said peptide introduced by the substitution or the insertion is bound to the peptide moiety; (2) the hybrid enzyme described in (1), in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from the amino acid sequence of SEQ ID NO: 1; (3) the hybrid enzyme described in (2), in which the peptide has a property of being capable of binding to a material having binding ability to CRP; (4) the hybrid enzyme described in (1), in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from any one of SEQ ID NO: 2 through SEQ ID NO: 5; (5) the hybrid enzyme described in (1), in which the enzyme is a glucose-6-phosphate dehydrogenase (hereinafter sometimes referred to as G6PDH), a  $\beta$ -galactosidase or an alkaline phosphatase; and (6) the hybrid enzyme described in (1), in which the material having binding ability to the peptide is an antibody. As a method for measuring a trace amount of CRP in a sample by a homogeneous colorimetry using the above-mentioned hybrid enzyme, the present invention provides (7) a reagent for measurement of CPR comprising the enzyme described in any one of (1) through (6); (8) the reagent described in (7) further comprising an anti-CRP antibody; (9) a kit for measurement of CRP containing a reagent comprising the enzyme described in any one of (1) through (6); (10) the kit described in (9) further comprising an anti-CRP antibody; (11) a method for measurement of CRP which is characterized in using the enzyme described in any one of (1) through (6); (12) the method described in (11) further comprising using an anti-CRP antibody in combination; and (13) a method for measurement of CRP comprising bringing a sample containing CRP, the enzyme described in any one of (1) through (6) and an anti-CRP antibody into contact with one another, then determining an activity of the enzyme, and determining the amount of CRP in the sample based on the resulting enzyme activity.

Further, as G6PDH-containing hybrid enzyme having a similar enzyme activity also when a foreign peptide is inserted therein, and having a property that the enzyme activity can vary when a material having binding ability to the foreign peptide moiety is bound thereto, for measurement of a macromolecule material in a homogeneous system, the present invention provides (14) G6PDH having a peptide introduced into a specific position of G6PDH by insertion or substitution; (15) the enzyme described in (14), in which the specific position is a position at which the G6PDH activity can be maintained even in the insertion or substitution of a peptide having 6 or more amino acid residues; (16) the hybrid enzyme described in (14), in which the specific position is a position at which the G6PDH activity is modulated when a material having binding ability to the peptide introduced by insertion or substitution is bound to the peptide; (17) the enzyme described in (14), in which the specific position is any position selected from the group consisting of the Leu305 to Asp310, Glu362 and the C-terminal of the amino acid sequence of G6PDH represented by SEQ ID NO: 6; (18) the enzyme described in (14), in which the peptide is selected from the amino acid sequence of CRP; and (19) the enzyme described in (14), in which the peptide has a character that there is a material having binding ability specifically to the part of the hybrid enzyme in which the peptide is substituted or inserted. The present invention further provides (20) a reagent comprising the enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the hybrid enzyme described in any one of (14) through (19) by insertion or substitution; (21) a kit comprising the enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of (14) through (19) by insertion or substitution; (22) a method comprising using the hybrid enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the enzyme according to any one of (14) through (19) by insertion or substitution; (23) a method comprising using the enzyme described in any one of (14)



through (19) in combination with a material having binding ability to the peptide introduced into the enzyme described in any one of (14) through (19) by insertion or substitution, for measurement of a material containing the peptide; (24) a method comprising bringing the enzyme described in any one of (14) through (19), a sample containing a material containing the peptide introduced into the enzyme by insertion or substitution and a material having binding ability to the peptide into contact with one another, then measuring activity of the enzyme, and determining the amount of the material containing the peptide in the sample based on the resulting enzyme activity, for measurement of the material containing the peptide; (25) a reagent comprising the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; (26) a kit comprising the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; (27) a method comprising using the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; and (28) a method comprising bringing the enzyme described in any one of (14) through (19) into contact with a sample containing a material having binding ability to the peptide introduced into the enzyme by insertion or substitution, then measuring an activity of the enzyme, and determining the amount of the material having binding ability to said peptide in the sample based on the resulting enzyme activity, for measurement of a material having binding ability to the peptide.

In the present invention, in order to produce a reagent for detecting a certain material or a material having binding ability thereto, it has been discovered that (29) a gene coding for a hybrid enzyme comprising an amino acid sequence into which a foreign peptide is introduced by substitution or insertion at any position selected from the group consisting of the Leu305 to Asp310, Glu362 and the C-terminal of the amino

acid sequence of G6PDH represented by SEQ ID NO: 6 is useful. The present invention further provides (30) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (29) into a vector DNA; (31) a transformant or a transductant comprising the recombinant DNA described in (30); (32) a method for producing a protein having an enzyme activity of G6PDH and a property that the G6PDH activity is modulated when a material having binding ability to an amino acid sequence introduced into G6PDH by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (31), and collecting the protein; (33) a gene coding for a hybrid enzyme comprising an amino acid sequence into which an amino acid sequence, which can be cleaved with a restriction enzyme is introduced by substitution or insertion at any position selected from the group consisting of the Leu305 to Asp310 positions, the Glu362 position and the C-terminal of the amino acid sequence of G6PDH represented by SEQ ID NO: 6; and (34) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (33) into a vector DNA.

Still further, the present invention provides (35)  $\beta$ -galactosidase in which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced into a specific position of  $\beta$ -galactosidase by insertion or substitution; (36) the  $\beta$ -galactosidase described in (35), in which the specific position is a site selected from the position Ile280/Asp281 and Val796/Ser797 of an amino acid sequence of a  $\beta$ -galactosidase represented by SEQ ID NO: 30; (37) a gene coding for the hybrid enzyme described in (36); (38) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (37) into a vector DNA; (39) a transformant or a transductant comprising the recombinant DNA described in (38); and (40) a method for producing a protein having an enzyme activity of a  $\beta$ -galactosidase and a property that the  $\beta$ -galactosidase activity varies when a material having binding ability to an amino acid sequence introduced into the  $\beta$ -galactosidase by substitution

or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (39), and collecting the protein.

Furthermore, the present invention provides (41) alkaline phosphatase in which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced into a specific position of an alkaline phosphatase by insertion or substitution; (42) the hybrid enzyme described in (41), in which the specific position is a site selected from the position Lys167/168Cys, Cys168/169Tyr, Glu407/408Asp, Lys91/93Thr and Tyr169/177Lys of an amino acid sequence of an alkaline phosphatase represented by SEQ ID NO: 31; (43) a gene coding for the hybrid enzyme described in (42); (44) a novel recombinant DNA, which is characterized in inserting the hybrid enzyme gene described in (43) into a vector DNA; (45) a transformant or a transductant comprising the recombinant DNA described in (44); and (46) a method for producing a protein having an enzyme activity of an alkaline phosphatase and having a property that the alkaline phosphatase activity varies when a material having binding ability to an amino acid sequence introduced into the alkaline phosphatase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (45), and collecting the protein.

【0005】

#### 【The Embodiments of the Invention】

Origin enzymes used for the hybrid enzymes of the present invention may be any, as long as they are enzymes generally used. Examples thereof include adenosine deaminases, alkaline phosphatases,  $\alpha$ -amylases, bacterial luciferases,  $\beta$ -galactosidases,  $\beta$ -galactosidase fragments,  $\beta$ -lactamases, carbonic anhydrases, catalases, firefly luciferases, glucose oxidases, glucose-6-phosphate dehydrogenases, glucosidases, hexokinases, horseradish peroxidases, invertases, isocitrate dehydrogenases, lysozymes, malate dehydrogenases, micro peroxidases, 6-phosphofructases and xanthine oxidases.

Further, all enzymes can be used, as long as each amino acid sequence or DNA

sequence arrangement thereof is determinate or can be determined, and the enzyme activity varies when a part of the enzyme are substituted by a foreign peptide or a foreign peptide is inserted into a part thereof and a material having binding ability to the peptide is bound to the peptide. Among which, enzymes having high enzyme activity, ones having good stability and ones which can be assayed by the colorimetry are particularly preferred. Examples thereof include G6PDHs,  $\beta$ -galactosidases and alkaline phosphatases. Genes of these enzymes are available by cloning methods from genomes usually employed. Of course, already cloned genes or synthetic DNA can also be used. These enzymes may be derived from any, and each amino acid sequence thereof may be a sequence obtained by deletion, substitution or addition of one or more amino acids, as long as they have intrinsic enzyme activity.

The foreign peptide used for preparing the hybrid enzyme of the present invention may be any, as long as a material such as an antibody or a receptor having binding ability to the peptide is present. Donors thereof include, for example, biological substances such as CRP, IgG, IgA, IgM, C3, C4,  $\beta$  2 microglobulin and albumin, various cancer markers such as  $\alpha$ -fetoprotein, CA19-9, prostatic specific antigen (PSA) and carcinoembryonic antigen (CEA), various hormones such as insulin, human chorionic gonadotropin (hCG), albumin, streptolysin O (SLO), prolactin and thyroid stimulating hormone (TSH), various toxins such as streptolysin O (SLO), or various viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and human papilloma virus (HPV), which are generally assayed(measured) in immunoassay systems.

There is no particular limitation on the vector used in the recombinant DNA of the present invention, as long as it is a vector such as a plasmid vector and a bacterio phage vector, which can be replicated and inherited in various hosts of procaryotic cells and/or eucaryotic cells. Examples of such vectors include Escherichia coli-derived plasmids such as pBR322, pBR325, pUC12, pUC13 and pBluescript, yeast-derived

plasmids such as pSH19 and pSH15, and Bacillus subtilis-derived plasmids such as pUB110, pTP5 and pC194, which are generally available in this field. Further, examples of the phage vectors include bacteriophages such as  $\lambda$  phage, and viruses of animals and insects such as retroviruses, vaccinia viruses and nuclear polyhedrosis viruses.

The host cells used for the production of the hybrid enzymes of the present invention include bacteria (for example, Escherichia coli), yeast (for example, Saccharomyces), animal cells (for example, chinese hamster cell CHO) and insect cells (for example, BmN4).

Construction of recombinant DNA, and expression and purification of the hybrid enzyme for producing the hybrid enzymes of the present invention may be carried out by known methods, for example, methods described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory).

There is no particular limitation on an expression vector, as long as it can be replicated and inherited in various hosts of procaryotic cells and/or eucaryotic cells, and has the function of expressing genes the hybrid enzymes from cloned gene in various hosts of procaryotic cells and/or eucaryotic cells, that is to say, the function of producing the desired hybrid enzymes. For example, the vectors generally available in this field are preferably pBR322, pUC12, pUC13, pTrcHis, pTrc99A, pMAL-c2 and artificially modified products thereof (DNA fragments obtained by treating the vectors with appropriate restriction enzymes) when the host cell is ESCHERICHIA coli; pRS403, pRS404, pRS413, pRS414 and pYES2 when the host cell is yeast; plasmids pRSVneo ATCC37224, pSV2dhfr ATCC37145, pdBPV-MMTneo ATCC37224 and pSV2neo ATCC37149 when the host cell is an animal cell; and Autographica californica nuclear polyhedrosis virus (AcNPV) and Bombyx mori nuclear polyhedrosis virus (BmNPV) when the host cell is an insect cell.

In the following, the preparation of the hybrid enzyme of the present invention

is described as to the case that G6PDH is used as the original enzyme, a CRP-derived peptide as the foreign peptide, and Escherichia coli as the host cell.

The G6PDH gene can be obtained, for example, by the following method. That is to say, first, cells are harvested from a culture product of Leuconostoc mesenteroides by centrifugation according to a conventional method described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory), and genomic DNA is extracted. Operations are hereinafter conducted according to well-known techniques generally employed, which are described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory) and the like, unless otherwise specified. Using the above-mentioned genomic DNA as a template, an oligonucleotide primer having an N-terminal sequence and a C-terminal sequence of the G6PDH gene sequence shown in R. Levy et al., J. Biological Chemistry, 266, 13028- (1991), or a sequence upstream therefrom and a sequence downstream therefrom is added, and the polymerase strand reaction is conducted by using a DNA thermal cycler (Perkin Elmer) to specifically amplify a DNA fragment containing the G6PDH gene. The resulting DNA fragment is integrated into a vector DNA according to a conventional method, thereby obtaining recombinant DNA containing DNA coding for G6PDH.

Further, for connecting the foreign peptide to the G6PDH gene by insertion or substitution, any method may be used. For example, the method for preparing a deletion mutant using an exonuclease described in "Laboratory Manual Genetic Engineering" (edited by Seikan Muramatsu, the 3rd edition, pages 219 to 230, Maruzen), the method for introducing artificial mutation such as the Kunkel method, the cassette method or the method using the PCR, or the phosphorothioate method described in "DNA Cloning 1" (edited by D.M. Glover et al., 2nd Edition, pages 197 to 228, Takara), the gap double stranded DNA method and the MHT protocol can be used in combination with each other. The resulting DNA coding for the hybrid enzyme is

normally inserted into the vector DNA according to conventional method, thereby being able to obtain the recombinant DNA containing the DNA coding for the hybrid enzyme. Then, a method for preparing a restriction enzyme site in the G6PDH gene inserted into the plasmid, and connecting the foreign peptide by insertion or substitution is described below.

That is to say, using the G6PDH gene as a template, primers to which a site cleavable with a restriction enzyme such as BamH I (BamH I site) is added in order to insert the BamH I site into the 5'-terminal of base sequences coding for amino acid sequences on both sides of a insertion position of a foreign peptide in G6PDH, is subjected to the PCR, in combination with primers of base sequences coding for upstream part or downstream part from a position where a foreign peptide is to be inserted respectively, to amplify DNA fragments to which the BamH I site is inserted. Then, two kinds of fragments thus obtained are connected to each other on the vector, thereby being able to construct the G6PDH gene into which the BamH I site is inserted. The restriction enzyme site is cleaved with the restriction enzyme, thereby being able to insert any DNA fragment having a complementary sequence and the cleaved site at both ends thereof.

When any amino acid sequence is eliminated for inserting the peptide by substitution, both sides of the sequence to be eliminated are subjected to the same operation as described above. Thus, the G6PDH gene connected to the foreign peptide by substitution can be obtained.

When the recombinant DNA is constructed, secretory production can also be conducted as a fused protein of the hybrid enzyme and another protein or peptide. Further, the fused protein produced by secretion can also be cleaved with an appropriate protease or by chemical treatment to obtain the hybrid enzyme. Examples of the proteins to be fused include maltose binding proteins and glutathione S-transferase, and examples of the peptides to be fused include histidine tags and FLAG tags.

The basic vectors for the construction of the recombinant DNA include, for example, plasmid vectors such as pBR322 (J. G. Sutcliffe, Cold Spring Harbor Symposium, 43, 77 (1979)), pUC18/19 (C. Yanisch-Perron et al., Gene, 33, 102-119 (1985)), pBluescript IISK+ (STRATEGENE), pMAL-C2 (NEW England Biolabs), pTrc99A (Amersham Pharmacia), pKK223-3 (Amersham Pharmacia) and pET-11 (STRATEGENE), and bacteriophage vectors such as  $\lambda$  ENBL3 (STRATEGENE) and  $\lambda$  DASHII (Funakoshi).

A promoter used in the recombinant DNA may be any, as long as it functions in Escheria coli. Examples thereof include a lac promoter, a trp promoter, a T7 promoter and derivatives thereof. Further, the recombinant DNA may contain an initiation signal such as a liposome binding sequence functioning in E. coli and a terminator. It may further contain a selective marker gene such as an ampicillin-resistance gene or a tetracycline-resistant gene.

[0006]

Using the recombinant DNA thus constructed, E. coli is transformed or transduced to prepare a transformant or a transductant. E. coli includes M103, JA221, HB101, C600, XL1-Blue and JM109.

Methods for transforming or transducing the recombinant DNA into Escherichia coli include, for example, the method of Cohen et al. (Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972)) and the method of Hanahan et al. (J. Mol. Biol., 166, 557 (1983)). The recombinant DNA may be obtained from the recombinant DNA-containing transformant or transductant by a conventional method such as the alkali miniprep method.

The hybrid enzyme of the present invention can be produced by cultivating the transformant or transductant of the recombinant DNA prepared as described above. Media used include, for example, Luria-Bertani medium (Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory)), 2 $\times$ YT medium



(Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory)) and M9 medium (J. Miller, Exp. Mol. Genet., Cold Spring Harbor Laboratory, New York, page 431 (1972)). The pH of the medium is preferably from 5 to 8. The cultivation is conducted usually at 14 to 42°C, preferably at 28 to 39°C, for 3 to 24 hours, optionally with aeration or stirring. Further, an expression inducing reagent such as isopropyl- $\beta$ -D-1-thiogalactopyranoside, or an antibiotic such as ampicillin or chloramphenicol may be added as needed.

The hybrid enzyme of the present invention can be obtained in the following manner from the culture product obtained by the above-mentioned cultivation. That is to say, when the hybrid enzyme exists in a culture solution of the culture product, a culture filtrate or culture supernatant containing the hybrid enzyme is obtained by a conventional method such as filtration or centrifugation. On the other hand, when the hybrid enzyme exists in periplasms or cells of the cultivated transformant or transductant, the culture product is subjected to a conventional method such as filtration or centrifugation to collect the periplasms or cells, which is suspended in a proper buffer solution and the cells in this solution are disrupted by a conventional method such as ultrasonication, lysozyme treatment or freeze-thawing. Then, a crude extract solution containing the hybrid enzyme is obtained by a conventional method such as filtration or centrifugation.

The hybrid enzyme may be separated and purified from the thus obtained culture filtrate, culture supernatant or crude extract solution containing the hybrid enzyme of the present invention by a suitable combination of known separating and purifying methods. These known separating and purifying methods include methods mainly utilizing a difference in solubility such as salting-out and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion exchange chromatography, methods

utilizing a difference in hydrophobicity such as hydrophobic chromatography, methods utilizing a difference in isoelectric point such as isoelectric point electrophoresis, and methods utilizing specific affinity such as affinity chromatography.

The effect of the anti-CRP antibody to the enzyme activity of the resulting hybrid enzyme can be examined, for example, in the following manner. That is to say, the enzyme activity of the hybrid enzyme is assayed in the absence and presence of the anti-CRP antibody, respectively, to examine modulations in activity due to the binding of the anti-CRP antibody. To 6  $\mu$ l of a solution obtained by diluting each hybrid enzyme solution with 100 mM Tris/HCl buffer (pH 7.8) containing 1% bovine albumin, 3 mM magnesium chloride and 150 mM sodium chloride to about 1 U/ml, 150  $\mu$ l of 100 mM Tris/HCl buffer containing 3 mM magnesium chloride and 150 mM sodium chloride (pH 7.8, hereinafter referred to as buffer A for brevity), or an antibody solution obtained by diluting 100 times anti-CRP goat antibody with buffer A is added, followed by reaction at 37°C for 5 minutes. Then, 75  $\mu$ l of buffer A containing 10 mM glucose-6-phosphate (G6P) and 6 mM nicotinamide adenine dinucleotide (NAD) are added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes are determined as G6PDH activity.

[0007]

The foreign peptide inserted into the enzyme may be any, as long as it maintains the structure that a material having binding ability to the peptide can bind thereto, and its enzyme activity is maintained even by insertion of the peptide. However, as described in Antibodies A Laboratory Manual (Ed Harlow et al., pages 76-, Cold Spring Harbor Laboratory), the peptide to be inserted is required to have at least 6 or more sequential amino acid residues for maintaining its antigenicity. When the foreign peptide to be inserted is a CRP-derived peptide, all or a part of the whole amino acid sequence of CRP represented by SEQ ID NO: 1 is used. Of these, an amino acid sequence containing 6 to 50 sequential amino acid residues, for example, a sequential

amino acid sequence selected from Gln(1)-Asp(16), Glu(14)-Ala(24), Leu(22)-Ser(45), Thr(41)-Asn(61), Arg(47)-Ile(63), Lys(114)-Lys(121), Glu(130)-Glu(138), Ile(134)-Gly(148), Gln(137)-Leu(152), Glu(147)-Leu(152), Asp(3)-Ser(18), Leu(152)-Val(165), Val(165)-Gly(178), Leu(121)-Ser(132) and Arg(188)-Glu(197), is preferred. In particular, an amino acid sequence containing at least 6 or more sequential amino acid residues selected from sequences represented by SEQ ID NO: 2 (Asp(3)-Ser(18)), SEQ ID NO: 3 (Leu(152)-Gly(178)), SEQ ID NO: 4 (Leu(121)-Ser(132)) and SEQ ID NO: 5 (Arg(188)-Glu(197)) is more preferred. The peptide moiety introduced into the enzyme by insertion or substitution may contain a sequence other than the desired peptide, such as a restriction enzyme site, which is sometimes introduced in the course of preparing the hybrid enzyme.

In the present invention, the foreign peptide may be inserted into the origin enzyme of the hybrid enzyme at any position, as long as the enzyme activity is maintained also when the foreign peptide is inserted, and is modified when a material having binding ability to the inserted foreign peptide is bound to the peptide. The positions considered to be suitable therefor include a site exposed on a surface of the enzyme and a site where activation is influenced. The preferred position at which the foreign peptide is inserted into G6PDH as the origin enzyme was studied using the CRP-derived peptide. That is to say, we tried to insert the foreign peptide into the N-terminal, 32/33 (which means "between the 32nd and the 33rd", hereinafter the same), 37/38, 48/49, 66/67, 87/88, 139/140, 226/227, 294/295, 296/297, 302/303, 305-310, 362/363, 409/410 and the C-terminal of G6PDH. Results thereof show that the effective sites for CRP measurement at which the enzyme activity remains in the insertion of the peptide and is modulated by the anti-CRP antibody reaction are the N-terminal, 139/140, 302/303, 305/306, 306/307, 308/309, 309/310, 362/363 and the C-terminal, as shown in Table 1.

[0008]

【Table 1】

| Insertion Site | Activity in Peptide<br>Insertion | Modulations in Enzyme<br>Activity in Antibody<br>Reaction |
|----------------|----------------------------------|---|
| Lys32/Lys33    | -                                | -   |
| Gln37/Lys38    | -                                | -   |
| Gln48/Ala49    | +                                | -   |
| Phe66/Thr67    | +                                | -   |
| Val87/Thr88    | -                                | -   |
| Gly226/Tyr227  | -                                | -   |
| Ala296/Asp297  | -                                | -   |
| Leu305/Asp306  | +                                | +   |
| Asp306/Val307  | +                                | +   |
| Pro308/Ala309  | +                                | +   |
| Ala309/Asp310  | +                                | +   |
| Glu329/Gly330  | -                                | -   |
| Glu362/Gln363  | +                                | +   |
| Lys409/Lys410  | -                                | -   |
| C-terminal     | +                                | +   |

The partial substitution of the amino acid sequence of the enzyme by the foreign peptide means that amino acid residues at specific sites of the enzyme are substituted by the amino acid sequence of the foreign peptide. In this case, the amino acid residue of the peptide introduced by substitution may be either more or less than the amino acid residues eliminated from the enzyme, as long as the enzyme activity of the original enzyme is maintained also after substitution, and is modulated when a material having binding ability to the foreign peptide introduced by substitution is bound to the peptide. It is preferred that the number of the amino acid residues

eliminated is approximately the same as that of the amino acid residues introduced. Further, it is preferred that the number of the amino acid residues of the peptide introduced by substitution is approximately the same as that of the amino acid residues of the foreign peptide introduced into the above-mentioned enzyme. Still further, the origin enzyme may be substituted by the foreign peptide at any position, as long as the enzyme activity is maintained also after substitution, and is modulated when a material having binding ability to the foreign peptide introduced by substitution is bound to the peptide. The position at which the foreign peptide is introduced by substitution is selected, based on the position at which the foreign peptide can be inserted into the original enzyme of the hybrid enzyme, as shown above.

[0009]

In the present invention, the hybrid enzyme obtained as described above is used for qualitative analysis or quantitative analysis

The hybrid enzyme of the present invention is modulated in its enzyme activity according to the binding amount thereof, when a material having binding ability to the foreign peptide introduced by substitution or insertion is bound to the peptide. Accordingly, the presence and amount of the material having the binding ability to the foreign peptide can be detected by allowing a sample containing the binding material to react with the hybrid enzyme of the present invention, and assaying modulations in the enzyme activity. The use of the hybrid enzyme of the present invention and the material having the binding ability allows the presence or amount of the material containing the peptide to be detected, by utilizing that the amount of the material having the binding ability which is bound to the hybrid enzyme is modulated by competition of the peptide introduced into the hybrid enzyme by insertion or substitution and the substance containing the peptide to the material having the binding ability. This process comprises the steps of (1) bringing a sample containing a material to be analyzed, the hybrid enzyme of the present invention and the substance having the

binding ability to the foreign peptide introduced into the hybrid enzyme of the present invention by insertion or substitution in contact with one another to form a reaction mixture, (2) bringing the reaction mixture into contact with a substrate to the starting enzyme, and (3) monitoring changes in the enzyme activity of the hybrid enzyme according to the amount of the material to be analyzed existing in the reaction mixture. Step (2) can also be carried out after the reaction mixture is allowed to reach a steady state or an equilibrium state, and step (1) can be carried out sequentially or concurrently. In step (1), a sample containing the material having the binding ability to the peptide can be reacted with the hybrid enzyme, thereby detecting the presence and amount of the material having the binding ability to the peptide.

As described above, the use of the hybrid enzyme-containing reagent of the present invention allows the presence or amount of the anti-CRP antibody to be directly assayed. Further, the use of a reagent containing the anti-CRP antibody together with the hybrid enzyme of the present invention makes it possible to conduct the assay for indirectly detecting the presence or amount of CRP as an antigen, by binding competition of the hybrid enzyme and CRP to the anti-CRP antibody as a binding material.

The enzyme activity of the hybrid enzyme may be assayed in accordance with the method for assaying the activity of the original enzyme.

The G6PDHs used for preparing G6PDH-containing hybrid enzymes for assaying a macromolecule material in a homogeneous system, which can maintain the same enzyme activity also after insertion of the foreign peptide and be modulated in the enzyme activity when the material having binding ability to the foreign peptide moiety is bound to the foreign peptide moiety, include one having the amino acid sequence represented by SEQ ID NO: 6 or a sequence obtained by deletion, substitution or addition of one or more amino acids of the amino acid sequence, and one having G6PDH activity even though it is different origin. Preferred examples of the positions

at which the foreign peptides are inserted include the positions described above.

The foreign peptides include all the peptides described above. Of these, the peptides as described above are preferably used as CRP.

The hybrid enzyme prepared by using an enzyme other than the G6PDH, into which the foreign peptide containing as a part thereof a CRP-derived peptide is introduced by insertion or substitution, is also prepared in the same manner as described above. The position at which the foreign peptide is introduced by insertion or substitution may also be appropriately selected in the same manner. When the  $\beta$ -galactosidase is used as the enzyme, the position may also be selected, based on the descriptions of FEBS Letters, 434, 23-27 (1998) and FEBS Letters, 438, 267-271 (1998). When the alkaline phosphatase is used, the position may also be selected, based on a method described in Proc. Natl. Acad. Sci. U.S.A., 92 (1995).

The use of the thus obtained hybrid enzyme into which the foreign peptide is introduced allows the presence or the amount of the material having the binding ability to the foreign peptide to be directly assayed, and the use of the hybrid enzyme and the material having the binding ability to the foreign peptide such as an antibody in combination makes it possible to conduct the assay for indirectly detecting the presence or the amount of the macromolecule material containing the foreign peptide. These assays may be conducted, based on the assaying operations of CRP or the anti-CRP antibody described above. Further, the use of the hybrid enzyme allows the macromolecule material to be assayed with high sensitivity in the homogeneous system, and this method can also be applied to general-purpose automatic analyzers.

Examples are shown below for illustrating the invention in more detail, but the invention is not construed as being limited by descriptions given therein.

[0010]

[Example]

## EXAMPLE 1

### Construction Plasmid containing G6PDH gene

Five milliliters of LACTOBACILLI MRS BROTH (DIFICO) was inoculated with *Leuconostoc mesenteroides*, and shake cultured at 26°C for 16 hours to obtain a culture medium. Then, the culture medium was centrifuged at 4°C at 6000 rpm for 10 minutes, and harvested to obtain cells. The cells were suspended in a 10 mM tris (hydroxymethyl)aminomethane (Tris/HCl) buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) (hereinafter referred to as TE for brevity), and achromopeptitase was added so as to give a final concentration of 300 u/ml, followed by standing at 37°C for 2 hours. Then, SDS and Proteinase K were added so as to give final concentrations of 0.5% and 100  $\mu$ g/ml, respectively, and the resulting suspension was further allowed to stand at 37°C for 2 hours to conduct bacteriolysis. Genomic DNA of *Leuconostoc mesenteroides* was extracted as a donor of a G6PDH gene according to a conventional method described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory). Operations were hereinafter conducted according to well-known techniques generally employed, which were described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory) and the like, unless otherwise specified. Then, for obtaining the G6PDH gene, the polymerase strand reaction (hereinafter referred to as PCR for brevity) was conducted according to the following procedure. The above-mentioned genomic DNA (10 ng) was added as a template DNA, and 0.1 nmol of each oligonucleotide primer represented by SEQ ID NO: 7 and SEQ ID NO: 8, respectively, containing the N-terminal and C-terminal sequences of the G6PDH gene, respectively, was added. Then, the reaction cycle at 94°C for 30 seconds, at 65°C for 30 seconds and at 72°C for 4 minutes was repeated 25 times using a DNA thermal cycler (Perkin Elmer) to amplify DNA fragment. As a result, an about 1.5-kbp DNA fragment containing the G6PDH gene was specifically amplified. The resulting DNA fragment was ligated to an Eco



RV site of cloning vector pBluescript II KS+ (Stratagene) to construct plasmid pBSWG.

Then, cloning vector pUC18 was digested with Eco RI and Sal I, and then, an end thereof was made flush. The resulting DNA fragment was ligated to the end made flush to construct plasmid pUCG which could express the G6PDH gene. Further, the 750th from the N-terminal of the G6PDH gene, cytosine, was varied to thymine using the oligonucleotide primer represented by SEQ ID NO: 9 and Mutan-K (Takara Shuzo Co., Ltd.) according to the Kunkel method, thereby constructing plasmid pBSMG containing the G6PDH gene having no restriction enzyme Nco I recognition sequence at a position other than the N-terminal, without changing the amino acid sequence. This plasmid was digested with restriction enzymes Nco I and Pst I, and an about 1.5-kbp G6PDH gene was recovered. This gene was ligated to an about 2.7-kbp DNA fragment obtained by digesting plasmid pUCG with restriction enzymes Nco I and Pst I to construct plasmid pUCMG.

[0011]

## EXAMPLE 2

Construction of Recombinant DNA Coding for Fused Enzyme Having Human CRP-Derived Peptide Ligated between Pro308/Ala309 of G6PDH

Using plasmid pUCMG of Example 1 as a template, and using the oligonucleotide primer represented by SEQ ID NO: 7, and the oligonucleotide primer represented by SEQ ID NO: 10, in which a restriction enzyme Bam H I recognition sequence was added to the 5'-end side of an anti-sense strand sequence upstream from Pro308, the PCR was conducted to obtain an about 0.9-kbp DNA fragment containing a part from the N-terminal to Pro308 of the G6PDH gene, in which the restriction enzyme Bam H I recognition sequence was ligated to a downstream site. Similarly, using the oligonucleotide primer represented by SEQ ID NO: 11, in which a restriction enzyme Bam HI recognition sequence was added to the 5'-end side of an anti-sense chain sequence downstream from Ala309, and the oligonucleotide primer represented by SEQ

ID NO: 12 containing the C-terminal anti-sense strand sequence of the G6PDH gene, an about 0.6-kbp DNA fragment containing a part from Ala309 to the C-terminal of the G6PDH gene, in which the restriction enzyme BamH I recognition sequence was ligated to an upstream site was obtained.

The fragment of the N-terminal side was digested with restriction enzymes BamH I and Nco I, and the fragment of the C-terminal side was digested with restriction enzymes BamH I and Pst I, followed by ligation with an about 2.7-kbp DNA fragment obtained by digesting plasmid pUCMG with restriction enzyme Nco I and Pst I. Thus, recombinant pUCMG308B having a BamH I site sequence only at Pro308/Ala309 of the G6PDH gene was constructed. This recombinant was cleaved with restriction enzyme BamH I, and synthetic polynucleotides (a combination of SEQ ID NO: 13 and SEQ ID NO: 14, complementary with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 2 were ligated thereto to construct pUCMG308C1. Synthetic nucleotides (a combination of SEQ ID NO: 15 and SEQ ID NO: 16, and a combination of SEQ ID NO: 21 and SEQ ID NO: 22, complementary with each other) having DNA coding for a part (portion) of the amino acid sequence represented by SEQ ID NO: 3 were each ligated to construct pUCMG308C2 and pUCMG308C13. Further, synthetic nucleotides (a combination of SEQ ID NO: 17 and SEQ ID NO: 18, complementary with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 4 were ligated to construct pUCMG308C3, and synthetic nucleotides (a combination of SEQ ID NO: 19 and SEQ ID NO: 20, complementary with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 5 were ligated to construct pUCMG308C5. The respective synthetic nucleotides have the following complementary structures with each other:

【Chemical Formula 1】

5'-gatccgacatgtcgaggaaggctttgtgtttcccaaagagtcggatacttccg -3'

SEQ ID NO: 13

|   |               |
|---|---------------|
| 3'- gatccggaagtatccgactctttgggaaacacaaaagccttcctcgacatgtcg-5' | SEQ ID NO: 14 |
| 5'-gatccgtgctgtcaccagatgagattaacacccatctatcttggcgggg -3'      | SEQ ID NO: 15 |
| 3'- gatcccccgcgaagatagatggtgttaatctcatctggtgacagcacg-5'       | SEQ ID NO: 16 |
| 5'-gatccctgaagaagggatacactgtgggggcagaagcaagcg -3'             | SEQ ID NO: 17 |
| 3'- gatccgcttgcttctgccccacagtgtatcccttcttcagg-5'              | SEQ ID NO: 18 |
| 5'-gatcccgggcactgaagtatgaagtgaaggcgaag -3'                    | SEQ ID NO: 19 |
| 3'- gatccttcgccttgcaacttcatacttcagtgcccg-5'                   | SEQ ID NO: 20 |
| 5'-gatcctagtgggagacattggaaatgtgaacatgtgggactttgtgg -3'        | SEQ ID NO: 21 |
| 3'- gatcccaaaagtcccatgttcacattccaatgtctccactag-5'             | SEQ ID NO: 22 |

[0012]

### EXAMPLE 3

Expression and Extraction of Fused Enzymes G308C1, G308C2, G308C3, G308C5 and G308C13

Recombinant DNAs pUCMG308C1, pUCMG308C3, pUCMG308C5, pUCMG308C2 and pUCMG308C13 of Example 2 were transformed into *E. Coli* XL1-Blue. According to the method of Levy et al. (*Protein Science*, 1, 329- (1992)), the transformed *E. Coli* was shake cultured in LB medium (DIFCO) at 37°C for 16 hours to obtain a culture medium. The resulting culture medium was inoculated in LB medium to yield a final concentration of 2%, and shake cultured at 37°C for 5 hours. Then, IPTG (isopropyl-  $\beta$  -D-thiogalactopyranoside) was added to achieve a final concentration of 0.5 mM, thereby inducing expression, and the culture was incubated at 37°C for 16 hours, followed by centrifugation at 4°C at 6000 rpm for 10 minutes to

obtain the cells. The cells were suspended in a 10 mM Tris/HCl buffer containing 8% sucrose, 0.1% Triton-X and 50mM EDTA, and lysozyme was added thereto so as to give a final concentration of 33 mg/ml. The resulting product was allowed to stand at 37°C for 30 minutes, and then, insoluble material was removed by centrifugation at 15000 rpm for 20 minutes to obtain a solution of each of fused enzymes G308C1, G308C2, G308C3, G308C5 and G308C13. As a control, a wild type enzyme solution was also similarly obtained from plasmid pUCMG.

[0013]

#### EXAMPLE 4

Effect of Anti-CRP Antibody to Enzyme Activity of Fused Enzymes G308C1, G308C2, G308C3, G308C5 and G308C13

The activity of the fused enzyme solutions obtained in Example 3 was assayed in the absence and presence of an anti-CRP antibody, respectively, to examine modulations in enzyme activity due to the binding of the anti-CRP antibody. To 6  $\mu$ l of a 400-fold dilution of each fused enzyme solution with a 100 mM Tris/HCl buffer (pH 7.8) containing 1% bovine albumin, 3 mM magnesium chloride and 150 mM sodium chloride, 150  $\mu$ l of a 100 mM Tris/HCl buffer containing 3 mM magnesium chloride and 150 mM sodium chloride (pH 7.8, buffer A), or of a 100-fold antibody dilution of anti-CPR goat antibody with buffer A was added, followed by reaction at 37°C for 5 minutes. Then, 75  $\mu$ l of buffer A containing 10 mM glucose-6-phosphate (G6P) and 6 mM nicotinamide adenine dinucleotide (NAD) was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody to that in the absence of the antibody is shown in Table 2. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzymes G308C1, G308C2,

G308C3, G308C5 and G308C13 are decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzymes in which CRP peptides represented by SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 or a part thereof are inserted at Pro308/Ala309 of G6PDH, it is found that their enzyme activity is inhibited by the binding of the anti-CRP antibody.

[Table 2]

|                     | Activity Ratio |
|---------------------|----------------|
| Wild type           | 101%           |
| Fused Enzyme G308C1 | 86%            |
| G308C2              | 29%            |
| G308C3              | 36%            |
| G308C5              | 88%            |
| G308C13             | 39%            |

Activity ratio (%) = (G6PDH activity in the presence of antibody)/(G6PDH activity in the absence of antibody) X 100

#### EXAMPLE 5

##### Assay of Anti-CRP antibody Using Fused Enzyme G308C1

Modulations in the activity of fused enzyme G308C1 which are dependent on the amount of the anti-CRP antibody were examined. To 50  $\mu$ l of each of 100-fold, 1000-fold, 10000-fold and 100000-fold dilutions of the anti-CRP monoclonal antibody with buffer A, or to 50  $\mu$ l of buffer A, 100  $\mu$ l of a 3300-fold dilution of fused enzyme G308C1 diluted with buffer A was added, followed by reaction at 37°C for 5 minutes. Then, 75  $\mu$ l of buffer A containing 10 mM G6P and 6 mM NAD was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. Results thereof are shown in Fig. 1. As known from the results, a phenomenon is observed that the enzyme activity increases with a decrease in the amount of the anti-CRP antibody. That is to say, it is shown that the anti-CRP antibody can be assayed using

the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Pro308/Ala309 of G6PDH.

[0014]

#### EXAMPLE 6

##### Assay of CRP Using Fused Enzyme G308C1

It was examined whether the hybrid enzyme activity inhibited by the binding of the anti-CRP antibody is recovered with an increase in the CRP concentration. To 6  $\mu$ l of each of CRP solutions of various concentrations (0, 10, 20 and 40 mg/dl), 100  $\mu$ l of a 3300-fold dilution of a solution of fused enzyme G308C1 diluted with buffer A was added, followed by reaction at 37°C for 3 minutes. Then, 50  $\mu$ l of a 10000-fold dilution of the anti-CRP monoclonal antibody diluted with buffer A was added thereto. After further reaction at 37°C for 3 minutes, 75  $\mu$ l of buffer A containing 10 mM G6P and 6 mM NAD was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. As shown in Fig. 2, a phenomenon is observed that the activity is recovered with an increase in CRP concentration. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Pro308/Ala309 of G6PDH.

#### EXAMPLE 7

##### Assay of CRP Using Fused Enzymes G308C2, G308C3 and G308C5

The assay of CRP was tried in the same manner as with Example 6. Dilutions of fused enzymes G308C2, G308C3 and G308C5 were 5000-fold, 15000-fold and 2000-fold, respectively, and the dilution ratio of anti-CRP goat antibody was 8-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Figs. 3, 4 and 5. That is to say, it is shown that CRP can be assayed using the fused enzymes in which CRP peptides represented by SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 are each inserted at Pro308/Ala309 of

G6PDH.

[0015]

#### EXAMPLE 8

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Inserted at Asp306/Val307 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 23 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 24, recombinant pUCMG306B having a BamH I site only at Asp306/Val307 of the G6PDH gene was constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct recombinant DNA pUCMG306C1, in the same manner as with Example 2.

#### EXAMPLE 9

Assay of CRP Using Fused Enzyme G306C1

A solution of fused enzyme G306C1 was obtained in the same manner as with Example 3. Then, the assay of CRP was tried in the same manner as with Example 6. A 500-fold dilution of the fused enzyme solution was used, and the dilution ratio of anti-CRP monoclonal antibody was 3200-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Fig. 6. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Asp306/Val307 of G6PDH.

[0016]

#### EXAMPLE 10

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Inserted between Ala309/Asp310 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 25 and a combination of the oligonucleotide primers

represented by SEQ ID NO: 12 and SEQ ID NO: 26, recombinant pUCMG309B having a BamH I site only at Ala309/Asp310 of the G6PDH gene was constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 were ligated thereto to construct recombinant DNA pUCMG309C1, in the same manner as with Example 2.

#### EXAMPLE 11

##### Assay of CRP Using Fused Enzyme G309C1

A solution of fused enzyme G309C1 was obtained in the same manner as with Example 3. Then, the assay of CRP was tried in the same manner as with Example 6. A 2500-fold dilution of the fused enzyme solution and a 1000-fold dilution of anti-CRP monoclonal antibody were used, and the dilution ratio of anti-CRP monoclonal antibody was 10000-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Fig. 7. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Ala309/Asp310 of G6PDH.

[0017]

#### EXAMPLE 12

##### Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Added to C-Terminal of G6PDH

Using plasmid pBSMG, the oligonucleotide primer represented by SEQ ID NO: 27 and Mutan-K (Takara Shuzo Co., Ltd.), plasmid pBSMGCB having a restriction enzyme BamH I sequence added to the C-terminal of the G6PDH gene was constructed according to the Kunkel method. Then, recombinant pUCMGCB having the restriction enzyme BamH I sequence only at the C-terminal was constructed, and the synthetic oligonucleotide represented by SEQ ID NO: 13 was ligated thereto to construct recombinant DNA pUCMGCC1, in the same manner as with Examples 1 and 2.



### EXAMPLE 13

#### Effect of Anti-CRP Antibody to Enzyme Activity of Fused Enzyme GCC1

A solution of fused enzyme GCC1 was obtained in the same manner as with Example 3. Then, the effect at the time when the anti-CRP antibody was ligated to fused enzyme GCC1 was examined by the same procedure as with Example 4. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody to that in the absence of the antibody is shown in Table 3. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzyme GCC1 is decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is ligated to the C-terminal of G6PDH, it is found that its enzyme activity is inhibited by the binding of the anti-CRP antibody.

[Table 3]

|                   | Activity Ratio |
|-------------------|----------------|
| Wild type         | 101%           |
| Fused Enzyme GCC1 | 95%            |

Activity ratio (%) = (G6PDH activity in the presence of antibody)/(G6PDH activity in the absence of antibody) X 100

[0018]

### EXAMPLE 14

#### Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Ligated between Glu362/Gln363 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 28 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 29, recombinant pUCMG362B having a restriction enzyme BamH I sequence only at Glu362/Gln363 of the G6PDH gene was

constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 were ligated thereto to construct recombinant DNA pUCMG362C1, in the same manner as with Example 2.

#### EXAMPLE 15

##### Assay of Anti-CRP Antibody Using Fused Enzyme G362C1

A solution of fused enzyme G362C1 was obtained in the same manner as with Example 3. Then, modulations in the activity of fused enzyme G362C1 with the amount of the anti-CRP antibody were examined in the same manner as with Example 5. As a result, a phenomenon is observed that the activity is increased with an increase in the anti-CRP antibody concentration, as shown in Fig. 8. That is to say, it is shown that an anti-CRP antibody can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Glu362/Gln363 of G6PDH.

[0019]

[Effect of the Invention]

Using the hybrid enzyme of the present invention into which the foreign peptide is inserted, the presence and the amount of the material having the binding ability to the foreign peptide can be directly assayed. Further, when the hybrid enzyme and the material having the binding ability to the foreign peptide are used in combination, the presence and the amount of a macromolecule material containing the foreign peptide can be indirectly detected and assayed. Furthermore, the use of the hybrid enzyme of the present invention makes it possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry, which enables early diagnosis of inflammation in the tissue, and further early diagnosis of diseases. Moreover, various macromolecule materials can be easily assayed in homogeneous systems.

[0020]

[Sequencing List]

#### SEQUENCE LISTING

<110> WAKO PURE CHEMICAL INDUSTRIES, LTD.

<120> Hybrid Enzymes and Use Thereof

<130> WJ017

<140>

<141>

<160> 31

<170> PatentIn Ver. 2.1

<210> 1

<211> 206

<212> PRT

<213> Human

<400> 1

Gln Thr Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp

1

5

10

15

Thr Ser Tyr Val Ser Leu Lys Ala Pro Leu Thr Lys Pro Leu Lys Ala

20

25

30

Phe Thr Val Cys Leu His Phe Tyr Thr Glu Leu Ser Ser Thr Arg Gly

35

40

45

Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg Gln Asp Asn Glu Ile Leu

50

55

60

Ile Phe Trp Ser Lys Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser

65

70

75

80

Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val Ala Pro Val His Ile

85

90

95

Cys Thr Ser Trp Glu Ser Ala Ser Gly Ile Val Glu Phe Trp Val Asp

100

105

110

Gly Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly Tyr Thr Val Gly

115

120

125

Ala Glu Ala Ser Ile Ile Leu Gly Gln Glu Gln Asp Ser Phe Gly Gly

130

135

140

Asn Phe Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Gly Asn Val Asn

145

150

155

160

Met Trp Asp Phe Val Leu Ser Pro Asp Glu Ile Asn Thr Ile Tyr Leu

165

170

175

Gly Gly Pro Phe Ser Pro Asn Val Leu Asn Trp Arg Ala Leu Lys Tyr

180

185

190

Glu Val Gln Gly Glu Val Phe Thr Lys Pro Gln Leu Trp Pro

195

200

205

<210> 2

<211> 16

<212> PRT

<213> Human

<400> 2

Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp Thr Ser

1

5

10

15

<210> 3

<211> 27

<212> PRT

<213> Human

<400> 3

Leu Val Gly Asp Ile Gly Asn Val Asn Met Trp Asp Phe Val Leu Ser

1

5

10

15

Pro Asp Glu Ile Asn Thr Ile Tyr Leu Gly Gly

20

25

<210> 4

<211> 12

<212> PRT

<213> Human

<400> 4

Leu Lys Lys Gly Tyr Thr Val Gly Ala Glu Ala Ser

1

5

10

<210> 5

<211> 10

<212> PRT

<213> Human

<400> 5

Arg Ala Leu Lys Tyr Glu Val Gln Gly Glu

1

5

10

<210> 6

<211> 486

<212> PRT

<213> *Leuconostoc mesenteroides*

<400> 6

Met Val Ser Glu Ile Lys Thr Leu Val Thr Phe Phe Gly Gly Thr Gly

1

5

10

15

Asp Leu Ala Lys Arg Lys Leu Tyr Pro Ser Val Phe Asn Leu Tyr Lys

20

25

30

Lys Gly Tyr Leu Gln Lys His Phe Ala Ile Val Gly Thr Ala Arg Gln

35

40

45

Ala Leu Asn Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys

50

55

60

Asp Phe Thr Asp Asp Gln Ala Gln Ala Glu Ala Phe Ile Glu His Phe

65

70

75

80

Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Ala Val Leu

85

90

95

Lys Glu Ala Ile Glu Glu Ala Ala Asp Lys Phe Asp Ile Asp Gly Asn

100

105

110

Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala

115

120

125

Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Asp Thr Gly Tyr Asn Arg

130

135

140

Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Asp Thr Ala Ala Glu

145

150

155

160



Leu Gln Asn Asp Leu Glu Asn Ala Phe Asp Asp Asn Gln Leu Phe Arg

165

170

175

Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu

180

185

190

Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile

195

200

205

Lys Asn Val Gln Val Thr Leu Ser Glu Val Leu Gly Val Glu Glu Arg

210

215

220

Ala Gly Tyr Tyr Asp Thr Ala Gly Ala Leu Leu Asp Met Ile Gln Asn

225

230

235

240

His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser

245

250

255

Phe Thr Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala

260

265

270

Leu Lys Ile Tyr Asp Glu Ala Glu Val Asn Lys Tyr Phe Val Arg Ala

275

280

285

Gln Tyr Gly Ala Gly Asp Ser Ala Asp Phe Lys Pro Tyr Leu Glu Glu

290

295

300

Leu Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu

305

310

315

320

Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg

325

330

335

Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe

340

345

350

Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ala

355

360

365

Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu

370

375

380

Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asp Leu

385

390

395

400

Gly Trp Thr Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr

405

410

415

Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala

420

425

430

Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Ser

435

440

445

Ala Val Tyr Thr Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly

450

455

460

Ser Met Gly Pro Glu Ala Ser Asp Lys Leu Leu Ala Ala Asn Gly Asp

465

470

475

480

Ala Trp Val Phe Lys Gly

485

<210> 7

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 7

ataaggggta caccatgggtt tcagaaatca agacgttag

39

<210> 8

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 8

ttccccgggct ttaattaacc tttaaacacc

30

<210> 9

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 9

tggttggtta gctatggaaa aaccagaatc

30

<210> 10

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 10

taggatccag gtacgtctaa ttcttcaagg tatg

34

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 11

atggatccgc tgattctaaa aacaatacct tc

32

<210> 12

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 12

aagcttgcat gcctgcaggt tcccg

25

<210> 13

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 2, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 13

gatccgacat gtcgaggaag gcttttgtgt ttcccaaaga gtcggatact tccg 54

<210> 14

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 13

<400> 14

gatccggaag tatccgactc tttagggaaac acaaaagcct tcttcgacat gtcg 54

<210> 15

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 15

gatccgtgct gtcaccagat gagattaaca ccattctatct tggcgggg

48

<210> 16

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 15



<400> 16

gatccccgc caagatagat ggtgttaatc tcatctgggtg acagcacg

48

<210> 17

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 4, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 17

gatccctgaa gaagggatac actgtggggg cagaagcaag cg

42

<210> 18

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 17

<400> 18

gatccgcttg ctctgcccc cacagtgtat cccttcttca gg

42

<210> 19

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 5, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 19

gatcccgggc actgaagtat gaagtgaag gcgaag

36

<210> 20

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 19

<400> 20

gatccttcgc ctgcacttc atacttcagt gcccg

36

<210> 21

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 21

gatcctagt ggagacattg gaaatgtgaa catgtgggac ttigtg

47

<210> 22

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complementary DNA of Sequence 21

<400> 22

gatccacaa agtccacat gttcacattt ccaatgtctc ccactag

47

<210> 23

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 23

taggatccgt ctaattcttc aaggtatggc ttg

33

<210> 24

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 24

aaggatccgt acctgctgat tctaaaaaca atac

34

<210> 25

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<400> 25

ttggatccag caggtacgtc taattcttca ag

32

<210> 26

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 26

taggatccga ttctaaaaac aataccttca tcg

33

<210> 27

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 27

gggtgttttaa aggtggatcc taattaaagc ccgg

34

<210> 28

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 28

taggatcctt ctgaaccaaa gttaaactg cc

32

<210> 29

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 29

atggatccca agaagcacia gaagctgtct tg

32

<210> 30

<211> 1024

<212> PRT

<213> Escherichia coli

<400> 30

Met Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Arg Asp

1

5

10

15

Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro

20

25

30

Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro

35

40

45

Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg Phe Ala Trp Phe

50

55

60



Pro Ala Pro Glu Ala Val Pro Glu Ser Trp Leu Glu Cys Asp Leu Pro

65

70

75

80

Glu Ala Asp Thr Val Val Val Pro Ser Asn Trp Gln Met His Gly Tyr

85

90

95

Asp Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro Ile Thr Val Asn Pro

100

105

110

Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys Tyr Ser Leu Thr Phe

115

120

125

Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr Arg Ile Ile Phe

130

135

140

Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys Asn Gly Arg Trp Val

145

150

155

160

Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu Phe Asp Leu Ser Ala

165

170

175

Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val Met Val Leu Arg Trp

180

185

190

Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met Trp Arg Met Ser Gly

195

200

205

Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro Thr Thr Gln Ile Ser

210

215

220

Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp Phe Ser Arg Ala Val

225

230

235

240

Leu Glu Ala Glu Val Gln Met Cys Gly Glu Leu Arg Asp Tyr Leu Arg

245

250

255

Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln Val Ala Ser Gly Thr

260

265

270

Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg Gly Gly Tyr Ala Asp

275

280

285

Arg Val Thr Leu Arg Leu Asn Val Glu Asn Pro Lys Leu Trp Ser Ala

290

295

300

Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu Leu His Thr Ala Asp

305 310 315 320

Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val Gly Phe Arg Glu Val

325 330 335

Arg Ile Glu Asn Gly Leu Leu Leu Leu Asn Gly Lys Pro Leu Leu Ile

340 345 350

Arg Gly Val Asn Arg His Glu His His Pro Leu His Gly Gln Val Met

355 360 365

Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu Met Lys Gln Asn Asn

370 375 380

Phe Asn Ala Val Arg Cys Ser His Tyr Pro Asn His Pro Leu Trp Tyr

385 390 395 400

Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val Asp Glu Ala Asn Ile

405 410 415

Glu Thr His Gly Met Val Pro Met Asn Arg Leu Thr Asp Asp Pro Arg

420 425 430

Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg Met Val Gln Arg Asp

435

440

445

Arg Asn His Pro Ser Val Ile Ile Trp Ser Leu Gly Asn Glu Ser Gly

450

455

460

His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp Ile Lys Ser Val Asp

465

470

475

480

Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly Ala Asp Thr Thr Ala

485

490

495

Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val Asp Glu Asp Gln Pro

500

505

510

Phe Pro Ala Val Pro Lys Trp Ser Ile Lys Lys Trp Leu Ser Leu Pro

515

520

525

Gly Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr Ala His Ala Met Gly

530

535

540

Asn Ser Leu Gly Gly Phe Ala Lys Tyr Trp Gln Ala Phe Arg Gln Tyr

545

550

555

560

Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp Val Asp Gln Ser Leu

565

570

575

Ile Lys Tyr Asp Glu Asn Gly Asn Pro Trp Ser Ala Tyr Gly Gly Asp

580

585

590

Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys Met Asn Gly Leu Val

595

600

605

Phe Ala Asp Arg Thr Pro His Pro Ala Leu Thr Glu Ala Lys His Gln

610

615

620

Gln Gln Phe Phe Gln Phe Arg Leu Ser Gly Gln Thr Ile Glu Val Thr

625

630

635

640

Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu Leu Leu His Trp Met

645

650

655

Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly Glu Val Pro Leu Asp

660

665

670

Val Ala Pro Gln Gly Lys Gln Leu Ile Glu Leu Pro Glu Leu Pro Gln

675

680

685

Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val Arg Val Val Gln Pro

690

695

700

Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile Ser Ala Trp Gln Gln

705

710

715

720

Trp Arg Leu Ala Glu Asn Leu Ser Val Thr Leu Pro Ala Ala Ser His

725

730

735

Ala Ile Pro His Leu Thr Thr Ser Glu Met Asp Phe Cys Ile Glu Leu

740

745

750

Gly Asn Lys Arg Trp Gln Phe Asn Arg Gln Ser Gly Phe Leu Ser Gln

755

760

765

Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr Pro Leu Arg Asp Gln

770

775

780

Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Ala Thr

785

790

795

800

Arg Ile Asp Pro Asn Ala Trp Val Glu Arg Trp Lys Ala Ala Gly His

805

810

815

Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr Ala Asp Thr Leu Ala

820

825

830

Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp Gln His Gln Gly Lys

835

840

845

Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile Asp Gly Ser Gly Gln

850

855

860

Met Ala Ile Thr Val Asp Val Glu Val Ala Ser Asp Thr Pro His Pro

865

870

875

880

Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln Val Ala Glu Arg Val

885

890

895

Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr Pro Asp Arg Leu Thr

900

905

910

Ala Ala Cys Phe Asp Arg Trp Asp Leu Pro Leu Ser Asp Met Tyr Thr

915

920

925

Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg Cys Gly Thr Arg Glu

930

935

940

Leu Asn Tyr Gly Pro His Gln Trp Arg Gly Asp Phe Gln Phe Asn Ile

945

950

955

960

Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr Ser His Arg His Leu

965

970

975

Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile Asp Gly Phe His Met

980

985

990

Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Phe

995

1000

1005

Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys

1010

1015

1020



<210> 31

<211> 448

<212> PRT

<213> Escherichia coli

<400> 31

Thr Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile

1 5 10 15

Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala Ala

20 25 30

Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu

35 40 45

Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr

50 55 60

Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu

65 70 75 80

Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro

85 90 95

68

Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly

100

105

110

Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp

115

120

125

His Pro Thr Ile Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr Gly

130

135

140

Asn Val Ser Thr Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala Leu Val

145

150

155

160

Ala His Val Thr Ser Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu

165

170

175

Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr

180

185

190

Glu Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala

195

200

205

Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr

210

215

220

Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala

225

230

235

240

Ala Ser Leu Asn Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu

245

250

255

Gly Leu Phe Ala Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro Lys

260

265

270

Ala Thr Tyr His Gly Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro

275

280

285

Asn Pro Gln Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp

290

295

300

Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln

305

310

315

320

Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys

325

330

335

Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala

340

345

350

Leu Glu Phe Ala Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala

355

360

365

Asp His Ala His Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala Pro

370

375

380

Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp Gly Ala Val Met Val Met

385

390

395

400

Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Gln Leu

405

410

415

Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr

420

425

430

Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys

435

440

445

[0021]

**[Brief Description of the Drawings]**

**[Fig. 1]** Fig.1 is a graph showing the ability for measuring an anti-CRP antibody of hybrid enzyme G308C1 of the present invention.

**[Fig. 2]** Fig.2 is a graph showing the ability for measuring CRP of hybrid enzyme G308C1 of the present invention. In Figs. 2 to 8,

Recovery (%) = {(G6PDH activity at each CRP concentration) – (G6PDH activity at 0 mg/dl of CRP)} / {(G6PDH activity in the absence of antibody) – (G6PDH activity at 0 mg/dl of CRP)} X 100; and

Activity ratio (%) = {G6PDH activity in the presence of antibody / G6PDH activity in the absence of antibody} X 100.

**[Fig. 3]** Fig.3 is a graph showing the ability for measuring CRP of hybrid enzyme G308C2 of the present invention.

**[Fig. 4]** Fig.4 is a graph showing the ability for measuring CRP of hybrid enzyme G308C3 of the present invention.

**[Fig. 5]** Fig.5 is a graph showing the ability for measuring CRP of hybrid enzyme G308C5 of the present invention.

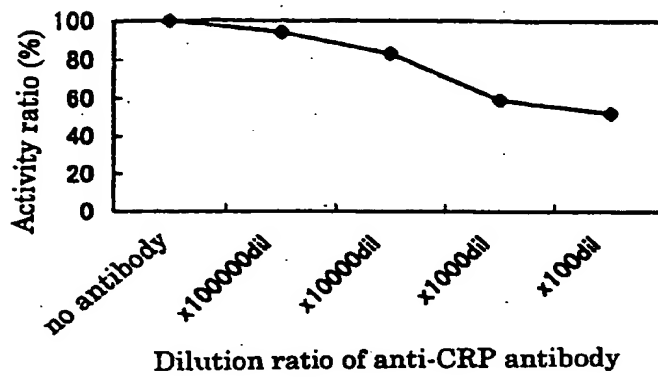
**[Fig. 6]** Fig.6 is a graph showing the ability for measuring CRP of hybrid enzyme G306C1 of the present invention.

**[Fig. 7]** Fig.7 is a graph showing the ability for measuring CRP of hybrid enzyme G309C1 of the present invention.

**[Fig. 8]** Fig.8 is a graph showing the ability for measuring the anti-CRP of hybrid enzyme G362C1 of the present invention.

[Fig.1]

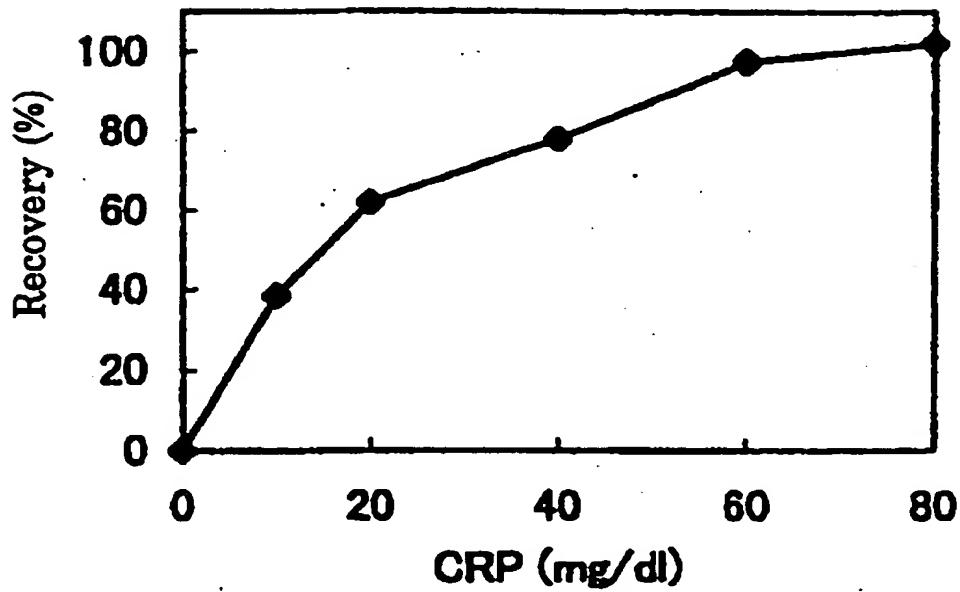
FIG.1



$$\text{Activity ratio (\%)} = \frac{\text{G6PDH activity in the presence of antibody}}{\text{G6PDH activity in the absence of antibody}} \times 100$$

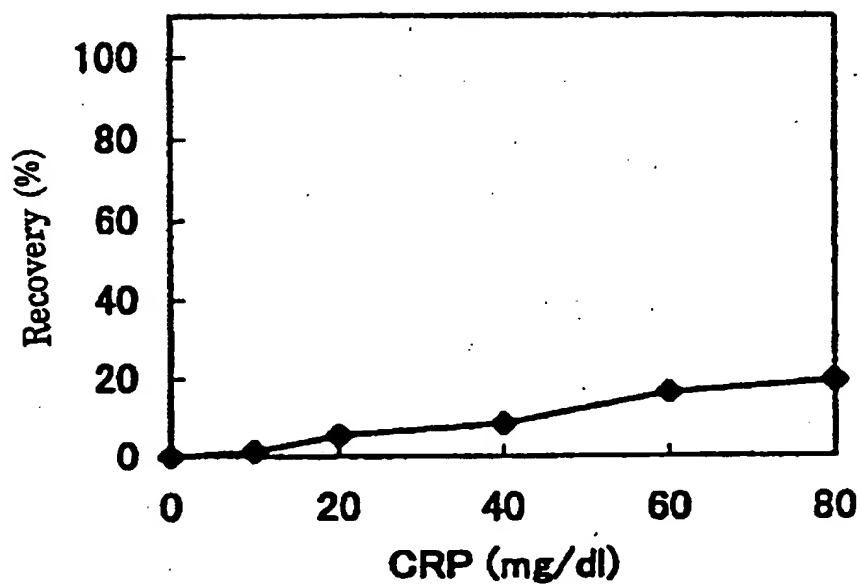
[Fig.2]

FIG.2



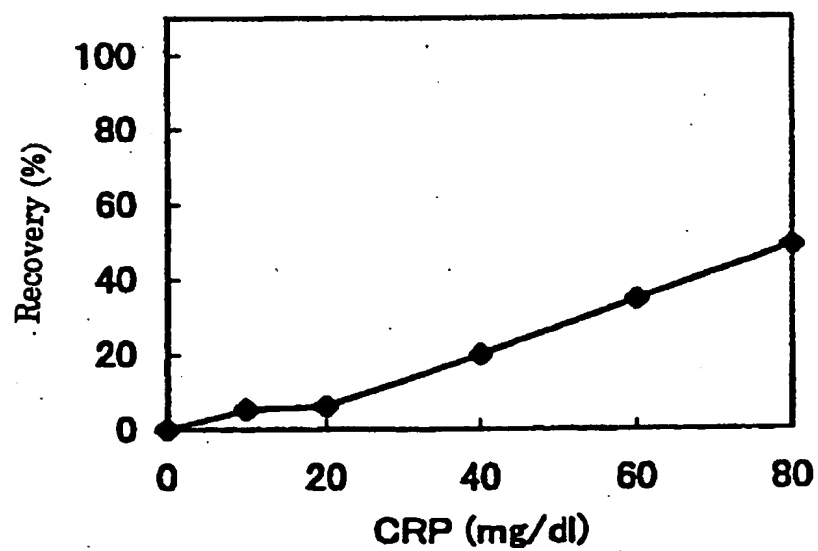
[Fig.3]

FIG.3



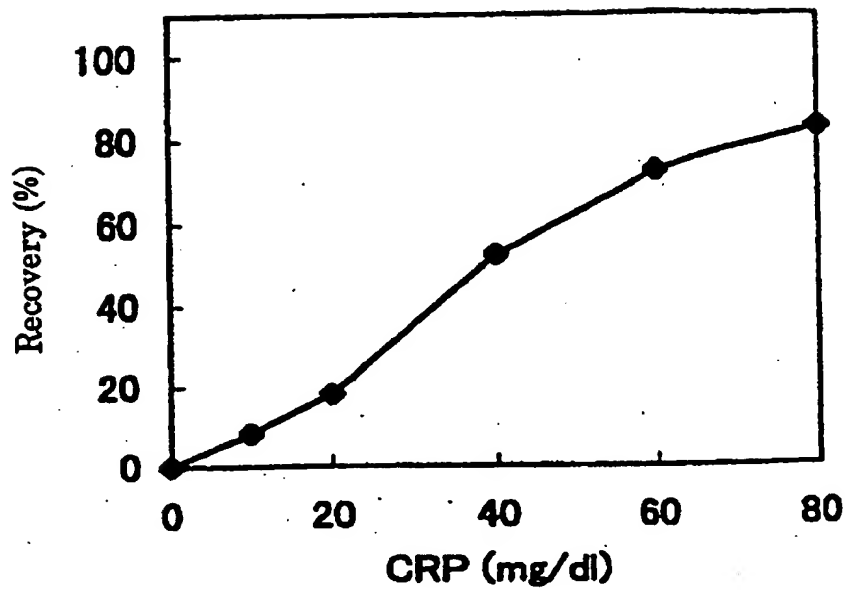
[Fig.4]

FIG.4



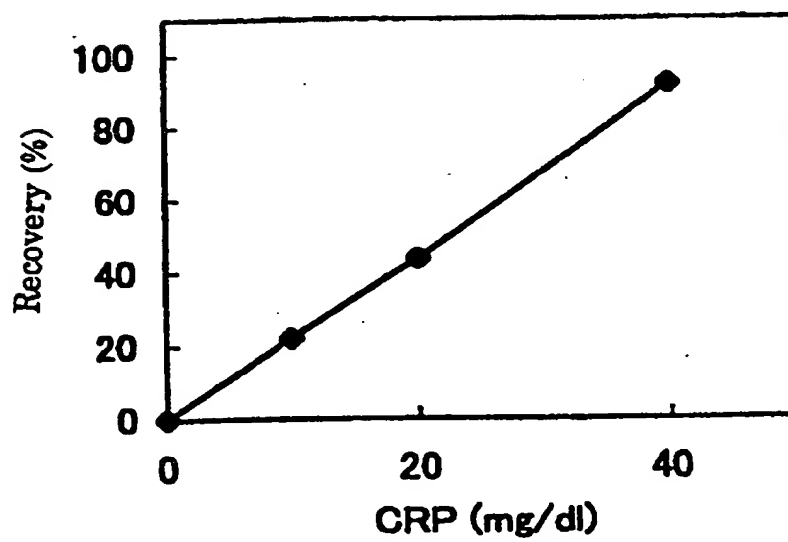
[Fig.5]

FIG.5



[Fig.6]

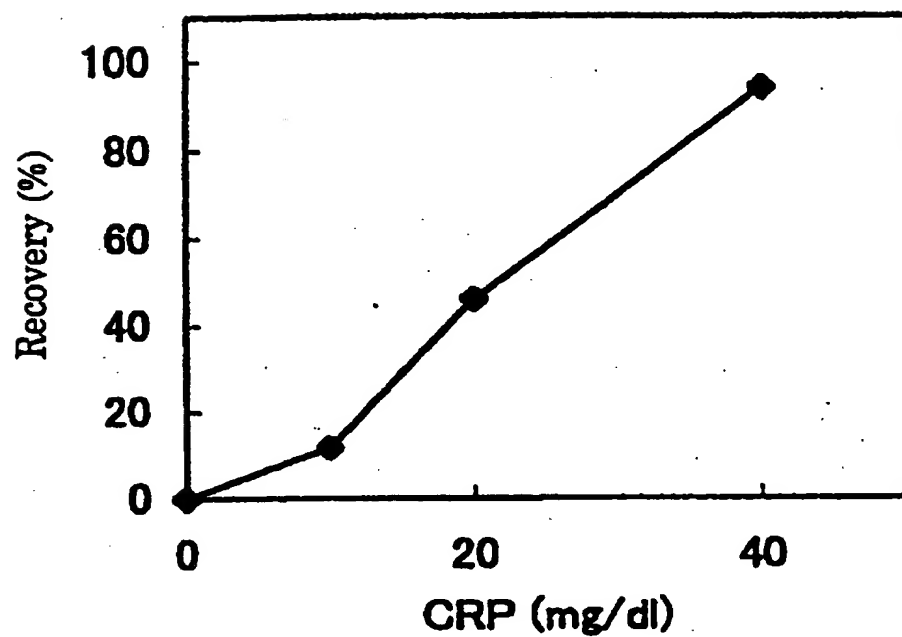
FIG.6





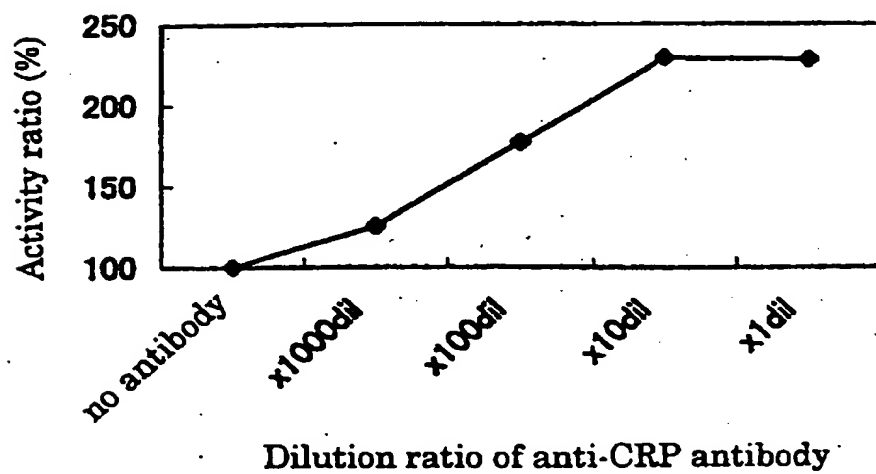
[Fig.7]

FIG.7



[Fig.8]

FIG.8



【Title of Document】 Abstract

【Abstract】

【Subject】 To provide is a method which makes it possible to measure a trace amount of CRP in a sample by a homogeneous colorimetry and a method for assaying a macromolecule material in a homogeneous system.

【Means for Solution】 The present inventors have found the specific positions that the enzyme activity is maintained also when the foreign peptide is inserted in that position, and is modified when a material having binding ability to the inserted foreign peptide is bound to the peptide. The present invention provide the hybrid enzyme which is inserted a CRP-derived peptide into a specific position. By determining the modulation of enzyme activity after contacting the hybrid enzyme with samples, it becomes possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry.

【Selected Drawing】 Non

RECOGNITION · ADDITIONAL INFORMATION

APPLICATION NUMBER No.174604/2000  
RECEPTION NUMBER No.50000722895  
NAME OF DOCUMENT Patent Request  
EXAMINER IN CHARGE Yuriko Nakamura 1730  
DATE OF EXECUTION June 16, 2000

<RECOGNITION · ADDITIONAL INFORMATION>

[DATE OF FILING] June 12, 2000

[APPLICANT FOR THE APPLICATION]

[IDENTIFICATION NUMBER] 000252300

[DOMICILE] 1-2, Doshomachi 3-Chome, Chuo-Ku,  
Osaka

[NAME] Wako Pure Chemical Industries, Ltd.

[AGENT] Applicant

[IDENTIFICATION NUMBER] 100089314

[DOMICILE] New Eiwa building 3-2, Nishishinbashi 2-  
Chome, Minato-Ku, Tokyo Ottawa Patent  
Office

[NAME] Akitoshi Otawa

[AGENT]

[IDENTIFICATION NUMBER] 100086999

[DOMICILE] New Eiwa building 3-2, Nishishinbashi 2-  
Chome, Minato-Ku, Tokyo Ottawa Patent  
Office

[NAME] Akiko Otaw

INFORMATION OF APPLICANT'S PERSONAL HISTORY

IDENTIFICATION NUMBER

[ 0 0 0 2 5 2 3 0 0 ]

1. DATE OF ALTERATION

August 7,1990

[REASON OF ALTERATION]

New registration

ADDRESS

1-2, Doshomachi 3-chome, Chuo-ku,

Osaka-shi, Osaka

NAME

Wako Pure Chemical Industries, Ltd.